



USA

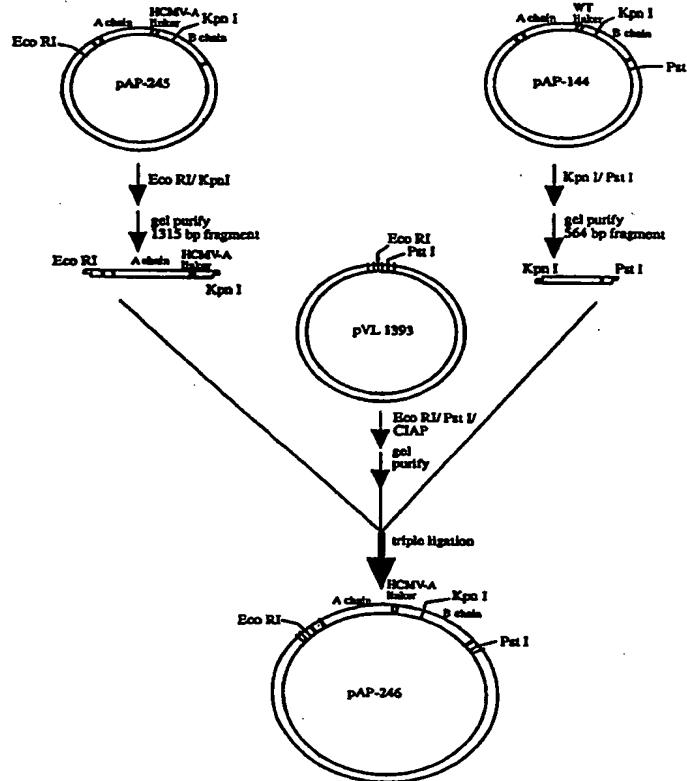
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ :		A2	(11) International Publication Number: WO 98/49311
C12N 15/29, 15/62, 15/70, 15/86, A61K 38/16, 48/00			(43) International Publication Date: 5 November 1998 (05.11.98)
(21) International Application Number: PCT/CA98/00394		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 30 April 1998 (30.04.98)			
(30) Priority Data:			
60/045,148	30 April 1997 (30.04.97)	US	
60/063,715	29 October 1997 (29.10.97)	US	
(71) Applicant (for all designated States except US): DE NOVO ENZYME CORPORATION [CA/CA]; #2 Suite SFU Discovery Park, Burnaby, British Columbia V5A 1S6 (CA).			
(72) Inventor; and			
(75) Inventor/Applicant (for US only): BORGFORD, Thor [CA/CA]; 443 Fadar Street, New Westminster, British Columbia V3L 3T2 (CA).			
(74) Agent: BERESKIN & PARR; 40th floor, 40 King Street West, Toronto, Ontario M5H 3Y2 (CA).			

(54) Title: RICIN-LIKE TOXIN VARIANTS FOR TREATMENT OF CANCER, VIRAL OR PARASITIC INFECTIONS

(57) Abstract

The present invention provides a protein having an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains. The linker sequence contains a cleavage recognition site for a disease specific protease such as a cancer, fungal, viral or parasitic protease. The invention also relates to a nucleic acid molecule encoding the protein and to expression vectors incorporating the nucleic acid molecule. Also provided is a method of inhibiting or destroying mammalian cancer cells, cells infected with a virus, a fungus, or parasite, or parasites utilizing the nucleic acid molecules and proteins of the invention and pharmaceutical compositions for treating human cancer, viral infection, fungal infection, or parasitic infection.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

- 1 -

Title: RICIN-LIKE TOXIN VARIANTS FOR TREATMENT OF CANCER, VIRAL OR PARASITIC INFECTIONS

FIELD OF THE INVENTION

The invention relates to proteins useful as therapeutics
5 against cancer, viral infections, parasitic and fungal infections. The
proteins contain A and B chains of a ricin-like toxin linked by a linker
sequence that is specifically cleaved and activated by proteases specific to
disease-associated pathogens or cells.

BACKGROUND OF THE INVENTION

10 Bacteria and plants are known to produce cytotoxic
proteins which may consist of one, two or several polypeptides or
subunits. Those proteins having a single subunit may be loosely
classified as Type I proteins. Many of the cytotoxins which have
evolved two subunit structures are referred to as type II proteins
15 (Saelinger, C.B. in Trafficking of Bacterial Toxins (eds. Saelinger, C.B.)
1-13 (CRC Press Inc., Boca Raton, Florida, 1990). One subunit, the A
chain, possesses the toxic activity whereas the second subunit, the B
chain, binds cell surfaces and mediates entry of the toxin into a target
cell. A subset of these toxins kill target cells by inhibiting protein
20 biosynthesis. For example, bacterial toxins such as diphtheria toxin or
Pseudomonas exotoxin inhibit protein synthesis by inactivating
elongation factor 2. Plant toxins such as ricin, abrin, and bacterial toxin
Shiga toxin, inhibit protein synthesis by directly inactivating the
ribosomes (Olsnes, S. & Phil, A. in Molecular action of toxins and
25 viruses (eds. Cohen, P. & vanHeyningen, S.) 51-105 Elsevier Biomedical
Press, Amsterdam, 1982).

Ricin, derived from the seeds of *Ricinus communis*
(castor oil plant), may be the most potent of the plant toxins. It is
estimated that a single ricin A chain is able to inactivate ribosomes at a

- 2 -

rate of 1500 ribosomes/minute. Consequently, a single molecule of ricin is enough to kill a cell (Olsnes, S. & Phil, A. in Molecular action of toxins and viruses (eds. Cohen, P. & vanHeyningen, S.) (Elsevier Biomedical Press, Amsterdam, 1982). The ricin toxin is a glycosylated heterodimer consisting of A and B chains with molecular masses of 30,625 Da and 31,431 Da linked by a disulphide bond. The A chain of ricin has an N-glycosidase activity and catalyzes the excision of a specific adenine residue from the 28S rRNA of eukaryotic ribosomes (Endo, Y. & Tsurugi, K. J., *Biol. Chem.* 262:8128 (1987)). The B chain of ricin, although not toxic in itself, promotes the toxicity of the A chain by binding to galactose residues on the surface of eukaryotic cells and stimulating receptor-mediated endocytosis of the toxin molecule (Simmons et al., *Biol. Chem.* 261:7912 (1986)). Once the toxin molecule consisting of the A and B chains is internalized into the cell via clathrin-dependent or independent mechanisms, the greater reduction potential within the cell induces a release of the active A chain, eliciting its inhibitory effect on protein synthesis and its cytotoxicity (Emmanuel, F. et al., *Anal. Biochem.* 173: 134-141 (1988); Blum, J.S. et al., *J. Biol. Chem.* 266: 22091-22095 (1991); Fiani, M.L. et al., *Arch. Biochem. Biophys.* 307: 225-230 (1993)). Empirical evidence suggests that activated toxin (e.g. ricin, shiga toxin and others) in the endosomes is transcytosed through the trans-Golgi network to the endoplasmic reticulum by retrograde transport before the A chain is translocated into the cytoplasm to elicit its action (Sandvig, K. & van Deurs, B., *FEBS Lett.* 346: 99-102 (1994)).

Protein toxins are initially produced in an inactive, precursor form. Ricin is initially produced as a single polypeptide (proricin) with a 35 amino acid N-terminal presequence and 12 amino acid linker between the A and B chains. The pre-sequence is removed during translocation of the ricin precursor into the endoplasmic reticulum (Lord, J.M., *Eur. J. Biochem.* 146:403-409 (1985) and Lord, J.M., *Eur. J. Biochem.* 146:411-416 (1985)). The proricin is then

- 3 -

translocated into specialized organelles called protein bodies where a plant protease cleaves the protein at a linker region between the A and B chains (Lord, J.M. et al., *FASAB Journal* 8:201-208 (1994)). The two chains, however, remain covalently attached by an interchain disulfide bond (cysteine 259 in the A chain to cysteine 4 in the B chain) and mature disulfide linked ricin is stored in protein bodies inside the plant cells. The A chain is inactive in proricin (O'Hare, M. et al., *FEBS Lett.* 273:200-204 (1990)) and it is inactive in the disulfide-linked mature ricin (Richardson, P.T. et al., *FEBS Lett.* 255:15-20 (1989)). The ribosomes of the castor bean plant are themselves susceptible to inactivation by ricin A chain; however, as there is no cell surface galactose to permit B chain recognition the A chain cannot re-enter the cell. The exact mechanism of A chain release and activation in target cell cytoplasm is not known (Lord, J.M. et al., *FASAB Journal* 8:201-208 (1994)). However, it is known that for activation to take place the disulfide bond between the A and B chains must be reduced and, hence, the linkage between subunits broken.

Diphtheria toxin is produced by *Corynebacterium diphtheriae* as a 535 amino acid polypeptide with a molecular weight of approximately 58kD (Greenfield, L. et al., *Proc. Natl. Acad. Sci. USA* 80:6853-6857 (1983); Pastan, I. et al., *Annu. Rev. Biochem.* 61:331-354 (1992); Collier, R.J. & Kandel, J., *J. Biol. Chem.* 246:1496-1503 (1971)). It is secreted as a single-chain polypeptide consisting of 2 functional domains. Similar to proricin, the N-terminal domain (A-chain) contains the cytotoxic moiety whereas the C-terminal domain (B-chain) is responsible for binding to the cells and facilitates toxin endocytosis. Conversely, the mechanism of cytotoxicity for diphtheria toxin is based on ADP-ribosylation of EF-2 thereby blocking protein synthesis and producing cell death. The 2 functional domains in diphtheria toxin are linked by an arginine-rich peptide sequence as well as a disulphide bond. Once the diphtheria toxin is internalized into the cell, the arginine-rich peptide linker is cleaved by trypsin-like enzymes and the

disulphide bond (Cys 186-201) is reduced. The cytotoxic domain is subsequently translocated into the cytosol substantially as described above for ricin and elicits ribosomal inhibition and cytotoxicity.

Pseudomonas exotoxin is also a 66kD single-chain toxin 5 protein secreted by *Pseudomonas aeruginosa* with a similar mechanism of cytotoxicity to that of diphtheria toxin (Pastan, I. et al., *Ann. Rev. Biochem.* 61:331-354 (1992); Ogata, M. et al., *J. Biol. Chem.* 267:25396-25401 (1992); Vagil, M.L. et al., *Infect. Immunol.* 16:353-361 (1977)). *Pseudomonas* exotoxin consists of 3 conjoint functional domains. The 10 first domain Ia (amino acids 1-252) is responsible for cell binding and toxin endocytosis, a second domain II (amino acids 253-364) is responsible for toxin translocation from the endocytic vesicle to the cytosol, and a third domain III (amino acids 400-613) is responsible for protein synthesis inhibition and cytotoxicity. After *Pseudomonas* 15 exotoxin enters the cell, the liberation of the cytotoxic domain is effected by both proteolytic cleavage of a polypeptide sequence in the second domain (near Arg 279) and the reduction of the disulphide bond (Cys 265-287) in the endocytic vesicles. In essence, the overall pathway to cytotoxicity is analogous to diphtheria toxin with the exception that the 20 toxin translocation domain in *Pseudomonas* exotoxin is structurally distinct.

Other toxins possessing distinct functional domains for cytotoxicity and cell binding/toxin translocation include abrin, modeccin and volvensin (Sandvig, K. et al., *Biochem. Soc. Trans.* 21:707-25 711 (1993)). Some toxins such as Shiga toxin and cholera toxin also have multiple polypeptide chains responsible for receptor binding and endocytosis.

The ricin gene has been cloned and sequenced, and the X-ray crystal structures of the A and B chains have been described 30 (Rutenber, E. et al. *Proteins* 10:240-250 (1991); Weston et al., *Mol. Bio.* 244:410-422, 1994; Lamb and Lord, *Eur. J. Biochem.* 14:265 (1985); Halling, K. et al. *Nucleic Acids Res.* 13:8019 (1985)). Similarly, the genes for

- 5 -

diphtheria toxin and *Pseudomonas* exotoxin have been cloned and sequenced, and the 3-dimensional structures of the toxin proteins have been elucidated and described (Columblatti, M. et al., *J. Biol. Chem.* 261:3030-3035 (1986); Allured, V.S. et al., *Proc. Natl. Acad. Sci. USA* 83:1320-1324 (1986); Gray, G.L. et al., *Proc. Natl. Acad. Sci. USA* 81:2645-2649 (1984); Greenfield, L. et al., *Proc. Natl. Acad. Sci. USA* 80:6853-6857 (1983); Collier, R.J. et al., *J. Biol. Chem.* 257:5283-5285 (1982)).

The potential of bacterial and plant toxins for inhibiting mammalian retroviruses, particularly acquired immunodeficiency syndrome (AIDS), has been investigated. Bacterial toxins such as *Pseudomonas* exotoxin-A and subunit A of diphtheria toxin; dual chain ribosomal inhibitory plant toxins such as ricin, and single chain ribosomal inhibitory proteins such as trichosanthin and pokeweed antiviral protein have been used for the elimination of HIV infected cells (Olson et al., *AIDS Res. and Human Retroviruses* 7:1025-1030 (1991)). The high toxicity of these toxins for mammalian cells, combined with a lack of specificity of action poses a major problem to the development of pharmaceuticals incorporating the toxins, such as immunotoxins.

Due to their extreme toxicity there has been much interest in making ricin-based immunotoxins as therapeutic agents for specifically destroying or inhibiting infected or tumourous cells or tissues (Vitetta et al., *Science* 238:1098-1104(1987)). An immunotoxin is a conjugate of a specific cell binding component, such as a monoclonal antibody or growth factor and the toxin in which the two protein components are covalently linked. Generally, the components are chemically coupled. However, the linkage may also be a peptide or disulfide bond. The antibody directs the toxin to cell types presenting a specific antigen thereby providing a specificity of action not possible with the natural toxin. Immunotoxins have been made both with the entire ricin molecule (i.e. both chains) and with the ricin A chain alone (Spooner et al., *Mol. Immunol.* 31:117-125, (1994)).

- 6 -

Immunotoxins made with the ricin dimer (IT-Rs) are more potent toxins than those made with only the A chain (IT-As). The increased toxicity of IT-Rs is thought to be attributed to the dual role of the B chains in binding to the cell surface and in translocating the A chain to the cytosolic compartment of the target cell (Vitetta et al., *Science* 238:1098-1104 (1987); Vitetta & Thorpe, *Seminars in Cell Biology* 2:47-58 (1991)). However, the presence of the B chain in these conjugates also promotes the entry of the immunotoxin into nontarget cells. Even small amounts of B chain may override the specificity of the cell-binding component as the B chain will bind nonspecifically to galactose associated with N-linked carbohydrates, which is present on most cells. IT-As are more specific and safer to use than IT-Rs. However, in the absence of the B chain the A chain has greatly reduced toxicity. Due to the reduced potency of IT-As as compared to IT-Rs, large doses of IT-As must be administered to patients. The large doses frequently cause immune responses and production of neutralizing antibodies in patients (Vitetta et al., *Science* 238:1098-1104 (1987)). IT-As and IT-Rs both suffer from reduced toxicity as the A chain is not released from the conjugate into the target cell cytoplasm.

A number of immunotoxins have been designed to recognize antigens on the surfaces of tumour cells and cells of the immune system (Pastan et al., *Annals New York Academy of Sciences* 758:345-353 (1995)). A major problem with the use of such immunotoxins is that the antibody component is its only targeting mechanism and the target antigen is often found on non-target cells (Vitetta et al., *Immunology Today* 14:252-259 (1993)). Also, the preparation of a suitable specific cell binding component may be problematic. For example, antigens specific for the target cell may not be available and many potential target cells and infective organisms can alter their antigenic make up rapidly to avoid immune recognition. In view of the extreme toxicity of proteins such as ricin, the lack of

specificity of the immunotoxins may severely limit their usefulness as therapeutics for the treatment of cancer and infectious diseases.

The insertion of intramolecular protease cleavage sites between the cytotoxic and cell-binding components of a toxin can mimic 5 the way that the natural toxin is activated. European patent application no. 466,222 describes the use of maize-derived pro-proteins which can be converted into active form by cleavage with extracellular blood enzymes such as factor Xa, thrombin or collagenase. Garred, O. et al. (*J. Biol. Chem.* 270:10817-10821 (1995)) documented the use of a ubiquitous 10 calcium-dependent serine protease, furin, to activate shiga toxin by cleavage of the trypsin-sensitive linkage between the cytotoxic A-chain and the pentamer of cell-binding B-units. Westby et al. (*Bioconjugate Chem.* 3:375-381 (1992)) documented fusion proteins which have a specific cell binding component and proricin with a protease sensitive 15 cleavage site specific for factor Xa within the linker sequence. O'Hare et al. (*FEBS Lett.* 273:200-204 (1990)) also described a recombinant fusion protein of RTA and staphylococcal protein A joined by a trypsin-sensitive cleavage site. In view of the ubiquitous nature of the extracellular proteases utilized in these approaches, such artificial 20 activation of the toxin precursor or immunotoxin does not confer a mechanism for intracellular toxin activation and the problems of target specificity and adverse immunological reactions to the cell-binding component of the immunotoxin remain.

In a variation of the approach of insertion of 25 intramolecular protease cleavage sites on proteins which combine a binding chain and a toxic chain, Leppla, S.H. et al. (*Bacterial Protein Toxins zbl.bakt.suppl.* 24:431-442 (1994)) suggest the replacement of the native cleavage site of the protective antigen (PA) produced by *Bacillus anthracis* with a cleavage site that is recognized by cells that contain a 30 particular protease. PA, recognizes, binds, and thereby assists in the internalization of lethal factor (LF) and edema toxin (ET). also produced by *Bacillus anthracis*. However, this approach is wholly dependent on

- 8 -

the availability of LF, or ET and PA all being localized to cells wherein the modified PA can be activated by the specific protease. It does not confer a mechanism for intracellular toxin activation and presents a problem of ensuring sufficient quantities of toxin for internalization in
5 target cells.

The *in vitro* activation of a *Staphylococcus*-derived pore-forming toxin, α -hemolysin by extracellular tumour-associated proteases has been documented (Panchel, R.G. et al., *Nature Biotechnology* 14:852-857 (1996)). Artificial activation of α -hemolysin *in*
10 *vitro* by said proteases was reported but the actual activity and utility of α -hemolysin in the destruction of target cells were not demonstrated.

Hemolysin does not inhibit protein synthesis but is a heptameric transmembrane pore which acts as a channel to allow leakage of molecules up to 3 kD thereby disrupting the ionic balances of
15 the living cell. The α -hemolysin activation domain is likely located on the outside of the target cell (for activation by extracellular proteases). The triggering mechanism in the disclosed hemolysin precursor does not involve the intracellular proteolytic cleavage of 2 functionally distinct domains. Also, the proteases used for the α -hemolysin
20 activation are ubiquitously secreted extracellular proteases and toxin activation would not be confined to activation in the vicinity of diseased cells. Such widespread activation of the toxin does not confer target specificity and limits the usefulness of said α -hemolysin toxin as therapeutics due to systemic toxicity.

A variety of proteases specifically associated with malignancy, viral infections and parasitic infections have been identified and described. For example, cathepsin is a family of serine, cysteine or aspartic endopeptidases and exopeptidases which has been implicated to play a primary role in cancer metastasis (Schwartz, M.K.,
30 *Clin. Chim. Acta* 237:67-78 (1995); Spiess, E. et al., *J. Histochem.*

- 9 -

Cytochem. 42:917-929 (1994); Scarborough, P.E. et al., *Protein Sci.* 2:264-276 (1993); Sloane, B.F. et al., *Proc. Natl. Acad. Sci. USA* 83:2483-2487 (1986); Mikkelsen, T. et al., *J. Neurosurgery* 83:285-290 (1995)). Matrix metalloproteinases (MMPs or matrixins) are zinc-dependent proteinases consisting of collagenases, matrilysin, stromelysins, gelatinases and macrophage elastase (Krane, S.M., *Ann. N.Y. Acad. Sci.* 732:1-10 (1994); Woessner, J.F., *Ann. N.Y. Acad. Sci.* 732:11-21 (1994); Carvalho, K. et al., *Biochem. Biophys. Res. Comm.* 191:172-179 (1993); Nakano, A. et al. *J. of Neurosurgery*, 83:298-307 (1995); Peng, K-W, et al. *Human Gene Therapy*, 8:729-738 (1997); More, D.H. et al. *Gynaecologic Oncology*, 65:78-82 (1997)). These proteases are involved in pathological matrix remodeling. Under normal physiological conditions, regulation of matrixin activity is effected at the level of gene expression. Enzymatic activity is also controlled stringently by tissue inhibitors of metalloproteinases (TIMPs) (Murphy, G. et al., *Ann. N.Y. Acad. Sci.* 732:31-41 (1994)). The expression of MMP genes is reported to be activated in inflammatory disorders (e.g. rheumatoid arthritis) and malignancy.

In malaria, parasitic serine and aspartic proteases are involved in host erythrocyte invasion by the *Plasmodium* parasite and in hemoglobin catabolism by intraerythrocytic malaria (O'Dea, K.P. et al., *Mol. Biochem. Parasitol.* 72:111-119 (1995); Blackman, M.J. et al., *Mol. Biochem. Parasitol.* 62:103-114 (1993); Cooper, J.A. et al., *Mol. Biochem. Parasitol.* 56:151-160 (1992); Goldberg, D.E. et al., *J. Exp. Med.* 173:961-969 (1991)). *Schistosoma mansoni* is also a pathogenic parasite which causes schistosomiasis or bilharzia. Elastinolytic proteinases have been associated specifically with the virulence of this particular parasite (McKerrow, J.H. et al., *J. Biol. Chem.* 260:3703-3707 (1985)).

Welch, A.R. et al. (*Proc. Natl. Acad. Sci. USA* 88:10797-10800 (1991)) has described a series of viral proteases which are specifically associated with human cytomegalovirus, human herpesviruses, Epstein-Barr virus, varicella zoster virus-I. and

- 10 -

infectious laryngotracheitis virus. These proteases possess similar substrate specificity and play an integral role in viral scaffold protein restructuring in capsid assembly and virus maturation. Other viral proteases serving similar functions have also been documented for

5 human T-cell leukemia virus (Blaha, I. et al., *FEBS Lett.* 309:389-393 (1992); Pettit, S.C. et al., *J. Biol. Chem.* 266:14539-14547 (1991)), hepatitis viruses (Hirowatari, Y. et al., *Anal. Biochem.* 225:113-120 (1995); Hirowatari, Y. et al., *Arch. Virol.* 133:349-356 (1993); Jewell, D.A. et al., *Biochemistry* 31:7862-7869 (1992)), poliomyelitis virus (Weidner, J.R. et

10 al., *Arch. Biochem. Biophys.* 286:402-408 (1991)), and human rhinovirus (Long, A.C. et al., *FEBS Lett.* 258:75-78 (1989)).

Candida yeasts are dimorphic fungi which are responsible for a majority of opportunistic infections in AIDS patients (Holmberg, K. and Myer, R., *Scand. J. Infect. Dis.* 18:179-192 (1986)). Aspartic 15 proteinases have been associated specifically with numerous virulent strains of *Candida* including *Candida albican*, *Candida tropicalis*, and *Candida parapsilosis* (Abad-Zapatero, C. et al., *Protein Sci.* 5:640-652 (1996); Cutfield, S.M. et al., *Biochemistry* 35:398-410 (1995); Ruchel, R. et al, *Zentralbl. Bakteriol. Mikrobiol Hyg. I Abt. Orig. A.* 255:537-548 (1983); 20 Remold, H. et al., *Biochim. Biophys. Acta* 167:399-406 (1968)), and the levels of these enzymes have been correlated with the lethality of the strain (Schreiber, B, et al., *Diagn. Microbiol. Infect. Dis.* 3:1-5 (1985)).

SUMMARY OF THE INVENTION

The invention relates to novel recombinant toxic 25 proteins which are specifically toxic to diseased cells but do not depend for their specificity of action on a specific cell binding component. The recombinant proteins of the invention have an A chain of a ricin-like toxin linked to a B chain by a synthetic linker sequence which may be cleaved specifically by a protease localised in cells or tissues affected by a 30 specific disease to liberate the toxic A chain thereby selectively inhibiting or destroying the diseased cells or tissues. The term diseased

cells as used herein, includes cells affected by cancer, or infected by fungi, or viruses, including retroviruses, or parasites.

Toxin targeting using the recombinant toxic proteins of the invention takes advantage of the fact that many DNA viruses 5 exploit host cellular transport mechanisms to escape immunological destruction. This is achieved by enhancing the retrograde translocation of host major histocompatibility complex (MHC) type I molecules from the endoplasmic reticulum into the cytoplasm (Bonifacino, J.S., *Nature* 384: 405-406 (1996); Wiertz, E.J. et al., *Nature* 384: 432-438 (1996)). The 10 facilitation of retrograde transport in diseased cells by the virus can enhance the transcytosis and cytotoxicity of a recombinant toxic protein of the present invention thereby further reducing non-specific cytotoxicity and improving the overall safety of the product.

The recombinant toxic proteins of the present invention 15 may be used to treat diseases including various forms of cancer such as T- and B-cell lymphoproliferative diseases, ovarian cancer, pancreatic cancer, head and neck cancer, squamous cell carcinoma, gastrointestinal cancer, breast cancer, prostate cancer, non small cell lung cancer, malaria, and diverse viral disease states associated with infection with 20 human cytomegalovirus, hepatitis virus, herpes virus, human rhinovirus, infectious laryngotracheitis virus, poliomyelitis virus, or varicella zoster virus.

In one aspect, the present invention provides a purified 25 and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains. The linker sequence is not a native linker sequence of a ricin-like toxin, but rather a synthetic heterologous linker sequence containing a cleavage recognition site for a disease-specific protease. The A and or 30 the B chain may be those of ricin.

In an embodiment, of the invention the cleavage recognition site is the cleavage recognition site for a cancer-associated

- 12 -

protease. In particular embodiments, the linker amino acid sequence comprises SLLKSRMVPNFN or SLLIARRMPNFN cleaved by cathepsin B; SKLVQASASGVN or SSYLKASDAPDN cleaved by an Epstein-Barr virus protease; RPKPQQFFGLMN cleaved by MMP-3 (stromelysin);

5 SLRPLALWRSFN cleaved by MMP-7 (matrilysin); SPQGIAGQRNFN cleaved by MMP-9; DVDERDVRGFASFL cleaved by a thermolysin-like MMP; SLPLGLWAPNFN cleaved by matrix metalloproteinase 2(MMP-2) ; SLLIFRSWANFN cleaved by cathepsin L; SGVVIATVIVIT cleaved by cathepsin D; SLGPQGIWGQFN cleaved by matrix metalloproteinase

10 1(MMP-1); KKSPGRVVGGSV cleaved by urokinase-type plasminogen activator; PQGLLGAPGILG cleaved by membrane type 1 matrixmetalloproteinase (MT-MMP); HGPEGLRVGFYEDVMGRGHARLVHVEEPT cleaved by stromelysin 3 (or MMP-11), thermolysin, fibroblast collagenase and stromelysin-1;

15 GPQGLAGQRGIV cleaved by matrix metalloproteinase 13 (collagenase-3); GGSGQRGRKALE cleaved by tissue-type plasminogen activator(tPA); SLSALLSSDIFN cleaved by human prostate-specific antigen; SLPRFKIIGGFN cleaved by kallikrein (hK3); SLLGIAVPGNFN cleaved by neutrophil elastase; and FFKNIVTPRTPP cleaved by calpain

20 (calcium activated neutral protease). The nucleic acid sequences for ricin A and B chains with each of the linker sequences are shown in Figures 2D, 35C, 3D, 4D, 5D, 6D, 16D, 17D, 34C, 36C , 37C, 38C , 39C, 40C, 41C, 42C , 43C, 44C, 45C, 46C and 47C, respectively.

In another embodiment, the cleavage recognition site is

25 the cleavage recognition site for a protease associated with the malaria parasite, *Plasmodium falciparum*. In particular embodiments, the linker amino acid sequence comprises QVVQLQNYDEED; LPIFGESEDNDE; QVVTGEAISVTM; ALERTFLSFPTN or KFQDMLNISQHQ. The nucleic nucleotide sequences for ricin A and B

30 chains with each of the linker sequences are shown in Figures 7D, 8D, 9D, 10D, and 11D.

In another embodiment, the cleavage recognition site is the cleavage recognition site for a viral protease. The linker sequences preferably comprise the sequence Y-X-Y-A-Z wherein X is valine or leucine, Y is a polar amino acid, and Z is serine, asparagine or valine.

- 5 In particular embodiments, the linker amino acid sequence comprises SGVVNASCRLAN or SSYVKASVSPEN cleaved by a human cytomegalovirus protease; SALVNASSAHVN or STYLQASEKFKN cleaved by a herpes simplex 1 virus protease; SSILNASVPNFN cleaved by a human herpes virus 6 protease; SQDVNAVEASSN or
- 10 SVYLQASTGYGN cleaved by a varicella zoster virus protease; or SKYLQANEVITN cleaved by an infectious laryngotracheitis virus protease. The nucleic nucleotide sequences for ricin A and B chains with each of the linker sequences are shown in Figures 12D, 13D, 14D, 15D, 18D, 19D, 20D, and 22D.

- 15 In another embodiment, the cleavage recognition site is the cleavage recognition site for a hepatitis A viral protease. In particular embodiments, the linker amino acid sequence comprises SELRTQSFSNWN or SELWSQGIDDDN cleaved by a hepatitis A virus protease. The nucleic nucleotide sequences for ricin A and B chains with each of the linker sequences are shown in Figures 23D or 24D.

- 20 In another embodiment, the cleavage recognition site is the cleavage recognition site for a hepatitis C viral protease. In particular embodiments, the linker amino acid sequence comprises DLEVVTSTWVFN, DEMEECASHLFN, EDVVCCSMSYFN or
- 25 KGWRLLAPITAY cleaved by a hepatitis C virus protease. The nucleic nucleotide sequences for ricin A and B chains with each of the linker sequences are shown in Figures 30C, 31C, 32C and 33C.

- 30 In another embodiment, the cleavage recognition site is the cleavage recognition site for a *Candida* fungal protease. In particular embodiments, the linker amino acid sequence is SKPAKFRLNFn, SKPIEFFRLNFn or SKPAEFFALNFn cleaved by *Candida* aspartic

protease. The nucleic nucleotide sequences for ricin A and B chains with the first linker sequence are shown in Figures 25D.

The present invention also provides a plasmid incorporating the nucleic acid of the invention. In an embodiment, the 5 plasmid has the restriction map as shown in Figures 2A, 3A, 4A, 5A, 6A, 7A, 8A, 9A, 10A, 11A, 12A, 13A, 14A, 15A, 16A, 17A, 18A, 19A, 20A, 21A, 22A, 23A, 24A, or 25A.

In another embodiment, the present invention provides a baculovirus transfer vector incorporating the nucleic acid of the 10 invention. In particular embodiments, the invention provides a baculovirus transfer vector having the DNA sequence as shown in Figure 1.

In a further embodiment, the present invention provides a baculovirus transfer vector incorporating the nucleic acid of the 15 invention. In particular embodiments, the invention provides a baculovirus transfer vector having the restriction map as shown in Figures 2C, 3C, 4C, 5C, 6C, 7C, 8C, 9C, 10C, 11C, 12C, 13C, 14C, 15C, 16C, 17C, 18C, 19C, 20C, 21C, 22C, 23C, 24C, 25C, 30A, 31A, 32A, 33A, 34A, 35A, 36A, 37A, 38A, 39A, 40A, 41A, 42A, 43A, 44A, 45A, 46A, or 47A. or 20 having the DNA sequence as shown in Figure 1.

In a further aspect, the present invention provides a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence 25 contains a cleavage recognition site for a disease-specific protease (e.g.. a cancer, viral, parasitic, or fungal protease). The A and/or the B chain may be those of ricin. In an embodiment, the cleavage recognition site is the cleavage recognition site for a cancer, viral or parasitic protease substantially as described above. In a particular embodiment, the cancer 30 is T-cell or B-cell lymphoproliferative disease. In another particular embodiment, the virus is human cytomegalovirus, Epstein-Barr virus, hepatitis virus, herpes virus, human rhinovirus, infectious

- 15 -

laryngotracheitis virus, poliomyelitis virus, or varicella zoster virus. In a further particular embodiment, the parasite is *Plasmodium falciparum*.

In a further aspect, the invention provides a pharmaceutical composition for treating a fungal infection, such as *Candida*, in a mammal comprising the recombinant protein of the invention and a pharmaceutically acceptable carrier, diluent or excipient.

In yet another aspect, the invention provides a method of inhibiting or destroying cells affected by a disease, which cells are associated with a disease specific protease, including cancer or infection with a virus, fungus, or a parasite each of which has a specific protease, comprising the steps of preparing a recombinant protein of the invention having a heterologous linker sequence which contains a cleavage recognition site for the disease-specific protease and administering the recombinant protein to the cells. In an embodiment, the cancer is T-cell or B-cell lymphoproliferative disease, ovarian cancer, pancreatic cancer, head and neck cancer, squamous cell carcinoma, gastrointestinal cancer, breast cancer, prostate cancer, non small cell lung cancer. In another embodiment, the virus is human cytomegalovirus, Epstein-Barr virus, hepatitis virus, herpes virus, human rhinovirus, human T-cell leukemia virus, infectious laryngotracheitis virus, poliomyelitis virus, or varicella zoster virus. In another embodiment, the parasite is *Plasmodium falciparum*.

The present invention also relates to a method of treating a mammal with disease wherein cells affected by the disease are associated with a disease specific protease, including cancer or infection with a virus, fungus, or a parasite each of which has a specific protease by administering an effective amount of one or more recombinant proteins of the invention to said mammal.

Still further, a process is provided for preparing a pharmaceutical for treating a mammal with disease wherein cells

affected by the disease are associated with a disease specific protease, including cancer or infection with a virus, fungus, or a parasite each of which has a specific protease comprising the steps of preparing a purified and isolated nucleic acid having a nucleotide sequence 5 encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for the disease-specific protease; introducing the nucleic acid into a host cell; expressing the nucleic acid in the host cell to obtain a recombinant 10 protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains wherein the linker sequence contains the cleavage recognition site for the disease-specific protease; and suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient.

15 In an embodiment, a process is provided for preparing a pharmaceutical for treating a mammal with disease wherein cells affected by the disease are associated with a disease specific protease, including cancer or infection with a virus, fungus, or a parasite each of which has a specific protease comprising the steps of identifying a 20 cleavage recognition site for the protease; preparing a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains wherein the linker sequence contains the cleavage recognition site for the protease and suspending the protein in a 25 pharmaceutically acceptable carrier, diluent or excipient.

In a further aspect, the invention provides a pharmaceutical composition for treating for treating a mammal with disease wherein cells affected by the disease are associated with a disease specific protease, including cancer or infection with a virus, fungus, or a 30 parasite comprising the recombinant protein of the invention and a pharmaceutically acceptable carrier, diluent or excipient.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DESCRIPTION OF THE DRAWINGS

The invention will be better understood with reference to 10 the drawings in which:

Figure 1 shows the DNA sequence of the baculovirus transfer vector, pVL1393;

Figure 2A summarizes the cloning strategy used to generate the pAP-213 construct;

15 Figure 2B shows the nucleotide sequence of the Cathepsin B linker regions of pAP-213;

Figure 2C shows the subcloning of the Cathepsin B linker variant into a baculovirus transfer vector;

20 Figure 2D shows the DNA sequence of the pAP-214 insert containing ricin and the Cathepsin B linker;

Figure 3A summarizes the cloning strategy used to generate the pAP-215 construct;

Figure 3B shows the nucleotide sequence of the MMP-3 linker regions of pAP-215;

25 Figure 3C shows the subcloning of the MMP-3 linker variant into a baculovirus transfer vector;

Figure 3D shows the DNA sequence of the pAP-216 insert containing ricin and the MMP-3 linker;

30 Figure 4A summarizes the cloning strategy used to generate the pAP-217 construct;

Figure 4B shows the nucleotide sequence of the MMP-7 linker regions of pAP-217;

- 18 -

Figure 4C shows the subcloning of the MMP-7 linker variant into a baculovirus transfer vector;

Figure 4D shows the DNA sequence of the pAP-218 insert containing ricin and the MMP-7 linker;

5 Figure 5A summarizes the cloning strategy used to generate the pAP-219 construct;

Figure 5B shows the nucleotide sequence of the MMP-9 linker regions of pAP-219;

10 Figure 5C shows the subcloning of the MMP-9 linker variant into a baculovirus transfer vector;

Figure 5D shows the DNA sequence of the pAP-220 insert containing ricin and the MMP-9 linker.

Figure 6A summarizes the cloning strategy used to generate the pAP-221 construct;

15 Figure 6B shows the nucleotide sequence of the thermolysin-like MMP linker regions of pAP-221;

Figure 6C shows the subcloning of the thermolysin-like MMP linker variant into a baculovirus transfer vector.

20 Figure 6D shows the DNA sequence of the pAP-222 insert containing ricin and the thermolysin-like MMP linker;

Figure 7A summarizes the cloning strategy used to generate the pAP-223 construct;

Figure 7B shows the nucleotide sequence of the Plasmodium falciparum-A linker regions of pAP-223;

25 Figure 7C shows the subcloning of the Plasmodium falciparum-A linker variant into a baculovirus transfer vector;

Figure 7D shows the DNA sequence of the pAP-224 insert containing ricin and the Plasmodium falciparum-A linker;

30 Figure 8A summarizes the cloning strategy used to generate the pAP-225 construct;

Figure 8B shows the nucleotide sequence of the Plasmodium falciparum-B linker regions of pAP-225;

- 19 -

Figure 8C shows the subcloning of the Plasmodium falciparum-B linker variant into a baculovirus transfer vector;

Figure 8D shows the DNA sequence of the pAP-226 insert containing ricin and the Plasmodium falciparum-B linker;

5 Figure 9A summarizes the cloning strategy used to generate the pAP-227 construct;

Figure 9B shows the nucleotide sequence of the Plasmodium falciparum-C linker regions of pAP-227;

10 Figure 9C shows the subcloning of the Plasmodium falciparum-C linker variant into a baculovirus transfer vector;

Figure 9D shows the DNA sequence of the pAP-228 insert containing ricin and the Plasmodium falciparum-C linker;

Figure 10A summarizes the cloning strategy used to generate the pAP-229 construct;

15 Figure 10B shows the nucleotide sequence of the Plasmodium falciparum-D linker regions of pAP-229;

Figure 10C shows the subcloning of the Plasmodium falciparum-D linker variant into a baculovirus transfer vector;

20 Figure 10D shows the DNA sequence of the pAP-230 insert containing ricin and the Plasmodium falciparum-D linker;

Figure 11A summarizes the cloning strategy used to generate the pAP-231 construct;

Figure 11B shows the nucleotide sequence of the Plasmodium falciparum-E linker regions of pAP-231;

25 Figure 11C shows the subcloning of the Plasmodium falciparum-E linker variant into a baculovirus transfer vector;

Figure 11D shows the DNA sequence of the pAP-232 insert containing ricin and the Plasmodium falciparum-E linker;

30 Figure 12A summarizes the cloning strategy used to generate the pAP-233 construct;

Figure 12B shows the nucleotide sequence of the HSV-A linker regions of pAP-233;

- 20 -

Figure 12C shows the subcloning of the HSV-A linker variant into a baculovirus transfer vector;

Figure 12D shows the DNA sequence of the pAP-234 insert containing ricin and the HSV-A linker;

5 Figure 13A summarizes the cloning strategy used to generate the pAP-235 construct;

Figure 13B shows the nucleotide sequence of the HSV-B linker regions of pAP-235;

10 Figure 13C shows the subcloning of the HSV-B linker variant into a baculovirus transfer vector;

Figure 13D shows the DNA sequence of the pAP-236 insert containing ricin and the HSV-B linker;

Figure 14A summarizes the cloning strategy used to generate the pAP-237 construct;

15 Figure 14B shows the nucleotide sequence of the VZV-A linker regions of pAP-237;

Figure 14C shows the subcloning of the VZV-A linker variant into a baculovirus transfer vector;

20 Figure 14D shows the DNA sequence of the pAP-238 insert containing ricin and the VZV-A linker;

Figure 15A summarizes the cloning strategy used to generate the pAP-239 construct;

Figure 15B shows the nucleotide sequence of the VZV-B linker regions of pAP-239;

25 Figure 15C shows the subcloning of the VZV-B linker variant into a baculovirus transfer vector;

Figure 15D shows the DNA sequence of the pAP-240 insert containing ricin and the VZV-B linker;

30 Figure 16A summarizes the cloning strategy used to generate the pAP-241 construct;

Figure 16B shows the nucleotide sequence of the EBV-A linker regions of pAP-241;

- 21 -

Figure 16C shows the subcloning of the EBV-A linker variant into a baculovirus transfer vector;

Figure 16D shows the DNA sequence of the pAP-242 insert containing ricin and the EBV-A linker;

5 Figure 17A summarizes the cloning strategy used to generate the pAP-243 construct;

Figure 17B shows the nucleotide sequence of the EBV-B linker regions of pAP-243;

10 Figure 17C shows the subcloning of the EBV-B linker variant into a baculovirus transfer vector;

Figure 17D shows the DNA sequence of the pAP-244 insert containing ricin and the EBV-B linker;

Figure 18A summarizes the cloning strategy used to generate the pAP-245 construct;

15 Figure 18B shows the nucleotide sequence of the CMV-A linker regions of pAP-245;

Figure 18C shows the subcloning of the CMV-A linker variant into a baculovirus transfer vector;

20 Figure 18D shows the DNA sequence of the pAP-246 insert containing ricin and the CMV-A linker;

Figure 19A summarizes the cloning strategy used to generate the pAP-247 construct;

Figure 19B shows the nucleotide sequence of the CMV-B linker regions of pAP-247;

25 Figure 19C shows the subcloning of the CMV-B linker variant into a baculovirus transfer vector;

Figure 19D shows the DNA sequence of the pAP-248 insert containing ricin and the CMV-B linker.

30 Figure 20A summarizes the cloning strategy used to generate the pAP-249 construct;

Figure 20B shows the nucleotide sequence of the HHV-6 linker regions of pAP-249;

- 22 -

Figure 20C shows the subcloning of the HHV-6 linker variant into a baculovirus transfer vector;

Figure 20D shows the DNA sequence of the pAP-250 insert containing ricin and the HHV-6 linker;

5 Figure 21 shows the amino acid sequences of the wild type ricin linker and cancer protease-sensitive amino acid linkers contained in pAP-213 to pAP-222 and linkers pAP-241 to pAP-244;

Figure 22A summarizes the cloning strategy used to generate the pAP-253 construct;

10 Figure 22B shows the nucleotide sequence of the ILV linker regions of pAP-253;

Figure 22C shows the subcloning of the ILV linker variant into a baculovirus transfer vector;

15 Figure 22D shows the DNA sequence of the pAP-254 insert containing ricin and the ILV linker;

Figure 23A summarizes the cloning strategy used to generate the pAP-257 construct;

Figure 23B shows the nucleotide sequence of the HAV-A linker regions of pAP-257;

20 Figure 23C shows the subcloning of the HAV-A linker variant into a baculovirus transfer vector;

Figure 23D shows the DNA sequence of the pAP-258 insert containing ricin and the HAV-A linker;

25 Figure 24A summarizes the cloning strategy used to generate the pAP-255 construct;

Figure 24B shows the nucleotide sequence of the HAV-B linker regions of pAP-255;

Figure 24C shows the subcloning of the HAV-B linker variant into a baculovirus transfer vector;

30 Figure 24D shows the DNA sequence of the pAP-256 insert containing ricin and the HAV-B linker;

- 23 -

Figure 25A summarizes the cloning strategy used to generate the pAP-259 construct;

Figure 25B shows the nucleotide sequence of the CAN linker regions of pAP-259;

5 Figure 25C shows the subcloning of the CAN linker variant into a baculovirus transfer vector;

Figure 25D shows the DNA sequence of the pAP-260 insert containing ricin and the CAN linker;

10 Figure 26 shows the amino acid sequences of the wild type ricin linker and *Plasmodium falciparum* protease-sensitive amino acid linkers contained in linkers pAP-223 to pAP-232;

15 Figure 27 shows the amino acid sequences of the wild type ricin linker and the viral protease-sensitive amino acid linkers contained in pAP-233 to pAP-240, pAP-245-pAP-248, pAP-253 to pAP-258;

Figure 28 shows the amino acid sequences of the wild type ricin linker and the *Candida* aspartic protease-sensitive amino acid linker contained in pAP-259 to pAP-264;

20 Figure 29 describes an alternative mutagenesis and subcloning strategy to provide a baculovirus transfer vector containing the ricin-like toxin variant gene; and

Figure 30A summarizes the cloning strategy used to generate the pAP-262 construct;

25 Figure 30B shows the nucleotide sequence of the HCV-A linker region of pAP-262;

Figure 30C shows the DNA sequence of the pAP-262 insert;

Figure 30D shows the amino acid sequence comparison of mutant preroricin linker region HCV-A to wild type;

30 Figure 31A summarizes the cloning strategy used to generate the pAP-264 construct;

- 24 -

Figure 31B shows the nucleotide sequence of the HCV-B linker region of pAP-264;

Figure 31C shows the DNA sequence of the pAP-264 insert;

5 Figure 31D shows the amino acid sequence comparison of mutant prorocin linker region HCV-B to wild type;

Figure 32A summarizes the cloning strategy used to generate the pAP-266 construct;

10 Figure 32B shows the nucleotide sequence of the HCV-C linker region of pAP-266;

Figure 32C shows the DNA sequence of the pAP-266 insert;

Figure 32D shows the amino acid sequence comparison of mutant prorocin linker region HCV-C to wild type;

15 Figure 33A summarizes the cloning strategy used to generate the pAP-268 construct;

Figure 33B shows the nucleotide sequence of the HCV-D linker region of pAP-268;

20 Figure 33C shows the DNA sequence of the pAP-268 insert;

Figure 33D shows the amino acid sequence comparison of mutant prorocin linker region HCV-D to wild type;

Figure 34A summarizes the cloning strategy used to generate the pAP-270 construct;

25 Figure 34B shows the nucleotide sequence of the MMP-2 linker region of pAP-270;

Figure 34C shows the DNA sequence of the pAP-270 insert;

30 Figure 34D shows the amino acid sequence comparison of mutant prorocin linker region of MMP-2 to wild type;

Figure 35A summarizes the cloning strategy used to generate the pAP-272 construct;

- 25 -

Figure 35B shows the nucleotide sequence of the Cathepsin B (Site 2) linker region of pAP-272;

Figure 35C shows the DNA sequence of the pAP-272 insert;

5 Figure 35D shows the amino acid sequence comparison of mutant prorocin linker region of Cathepsin B (Site 2) to wild type;

Figure 36A summarizes the cloning strategy used to generate the pAP-274 construct;

10 Figure 36B shows the nucleotide sequence of the Cathepsin L linker region of pAP-274;

Figure 36C shows the DNA sequence of the pAP-274 insert;

Figure 36D shows the amino acid sequence comparison of mutant prorocin linker region of Cathepsin L to wild type;

15 Figure 37A summarizes the cloning strategy used to generate the pAP-276 construct;

Figure 37B shows the nucleotide sequence of the Cathepsin D linker region of pAP-276;

20 Figure 37C shows the DNA sequence of the pAP-276 insert;

Figure 37D shows the amino acid sequence comparison of mutant prorocin linker region of Cathepsin D to wild type;

Figure 38A summarizes the cloning strategy used to generate the pAP-278 construct;

25 Figure 38B shows the nucleotide sequence of the MMP-1 linker region of pAP-278;

Figure 38C shows the DNA sequence of the pAP-278 insert;

30 Figure 38D shows the amino acid sequence comparison of mutant prorocin linker region of MMP-1 to wild type;

Figure 39A summarizes the cloning strategy used to generate the pAP-280 construct;

- 26 -

Figure 39B shows the nucleotide sequence of the Urokinase-Type Plasminogen Activator linker region of pAP-280;

Figure 39C shows the DNA sequence of the pAP-280 insert;

5 Figure 39D shows the amino acid sequence comparison of mutant preproricin linker region of Urokinase-Type Plasminogen Activator to wild type;

Figure 40A summarizes the cloning strategy used to generate the pAP-282 construct;

10 Figure 40B shows the nucleotide sequence of the MT-MMP linker region of pAP-282;

Figure 40C shows the DNA sequence of the pAP-282 insert;

15 Figure 40D shows the amino acid sequence comparison of mutant preproricin linker region of MT-MMP to wild type;

Figure 41A summarizes the cloning strategy used to generate the pAP-284 construct;

Figure 41B shows the nucleotide sequence of the MMP-11 linker region of pAP-284;

20 Figure 41C shows the DNA sequence of the pAP-284 insert;

Figure 41D shows the amino acid sequence comparison of mutant preproricin linker region of MMP-11 to wild type;

25 Figure 42A summarizes the cloning strategy used to generate the pAP-286 construct;

Figure 42B shows the nucleotide sequence of the MMP-13 linker region of pAP-286;

Figure 42C shows the DNA sequence of the pAP-286 insert;

30 Figure 42D shows the amino acid sequence comparison of mutant preproricin linker region of MMP-13 to wild type;

Figure 43A summarizes the cloning strategy used to generate the pAP-288 construct;

Figure 43B shows the nucleotide sequence of the Tissue-type Plasminogen Activator linker region of pAP-288;

5 Figure 43C shows the DNA sequence of the pAP-288 insert;

Figure 43D shows the amino acid sequence comparison of mutant preproricin linker region of Tissue-type Plasminogen Activator to wild type;

10 Figure 44A summarizes the cloning strategy used to generate the pAP-290 construct;

Figure 44B shows the nucleotide sequence of the human Prostate-Specific Antigen linker region of pAP-290;

15 Figure 44C shows the DNA sequence of the pAP-290 insert;

Figure 44D shows the amino acid sequence comparison of mutant preproricin linker region of the human Prostate-Specific Antigen to wild type;

20 Figure 45A summarizes the cloning strategy used to generate the pAP-292 construct;

Figure 45B shows the nucleotide sequence of the kallikrein linker region of pAP-292;

Figure 45C shows the DNA sequence of the pAP-292 insert;

25 Figure 45D shows the amino acid sequence comparison of mutant preproricin linker region of the kallikrein to wild type;

Figure 46A summarizes the cloning strategy used to generate the pAP-294 construct;

30 Figure 46B shows the nucleotide sequence of the neutrophil elastase linker region of pAP-294;

Figure 46C shows the DNA sequence of the pAP-294 insert;

- 28 -

Figure 46D shows the amino acid sequence comparison of mutant preproricin linker region of neutrophil elastase to wild type;

Figure 47A summarizes the cloning strategy used to generate the pAP-296 construct;

5 Figure 47B shows the nucleotide sequence of the calpain linker region of pAP-296;

Figure 47C shows the DNA sequence of the pAP-296 insert;

10 Figure 47D shows the amino acid sequence comparison of mutant preproricin linker region of calpain to wild type;

Figure 48 is a blot showing cleavage of pAP-214 by Cathepsin B;

Figure 49 is a blot showing cleavage of pAP-220 with MMP-9;

15 Figure 50 is a blot showing activation of pAP-214; and Figure 51 is a blot showing activation of pAP-220.

Figure 52 is a blot showing cleavage of pAP-248 with HCMV.

Figure 53 is a blot showing activation of pAP-248.

20 Figure 54 is a blot showing cleavage of pAP-256 by HAV 3C.

Figure 55 is a blot showing activation of pAP-256.

25 Figure 56 is a semi-logarithmic graph illustrating the cytotoxicity to COS-1 cells of undigested pAP-214 and pAP-214 digested with Cathepsin B.

Figure 57 is a semi-logarithmic graph illustrating the cytotoxicity of pAP-220 digested with MMP-9 compared to freshly thawed pAP-220 and ricin on COS-1 cells.

30 Figure 58 is a blot showing cleavage of pAP-270 with MMP-2.

Figure 59 is a blot showing activation of pAP-270.

Figure 60 is a blot showing cleavage of pAP-288 by t-PA.

- 29 -

Figure 61 is a blot showing activation of pAP-288.

Figure 62 is a blot showing cleavage of pAP-294 with human neutrophil elastase.

5 Figure 63 is a blot showing activation of pAP-294.
Figure 64 is a blot showing cleavage of pAP-296 with calpain.

Figure 65 is a blot showing activation of pAP-296.

Figure 66 is a blot showing cleavage of pAP-222 with MMP-2.

10 Figure 67 is a blot showing activation of pAP-222.

DETAILED DESCRIPTION OF THE INVENTION

Nucleic Acid Molecules of the Invention

As mentioned above, the present invention relates to novel nucleic acid molecules comprising a nucleotide sequence 15 encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains. The heterologous linker sequence contains a cleavage recognition site for a disease-specific protease (e.g. a viral protease, parasitic protease, cancer-associated protease, or a fungal protease).

20 The term "isolated and purified" as used herein refers to a nucleic acid substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors, or other chemicals when chemically synthesized. An "isolated and purified" nucleic acid is also substantially free of 25 sequences which naturally flank the nucleic acid (*i.e.* sequences located at the 5' and 3' ends of the nucleic acid) from which the nucleic acid is derived. The term "nucleic acid" is intended to include DNA and RNA and can be either double stranded or single stranded.

The term "linker sequence" as used herein refers to an 30 internal amino acid sequence within the protein encoded by the nucleic acid molecule of the invention which contains residues linking the A and B chain so as to render the A chain incapable of exerting its toxic

- 30 -

effect, for example catalytically inhibiting translation of a eukaryotic ribosome. By heterologous is meant that the linker sequence is not a sequence native to the A or B chain of a ricin-like toxin or precursor thereof. However, preferably, the linker sequence may be of a similar 5 length to the linker sequence of a ricin-like toxin and should not interfere with the role of the B chain in cell binding and transport into the cytoplasm. When the linker sequence is cleaved the A chain becomes active or toxic.

The nucleic acid molecule of the invention is cloned by 10 subjecting a preproricin cDNA clone to site-directed mutagenesis in order to generate a series of variants differing only in the sequence between the A and B chains (linker region). Oligonucleotides, corresponding to the extreme 5' and 3' ends of the preproricin gene are synthesized and used to PCR amplify the gene. Using the cDNA 15 sequence for preproricin (Lamb et al., *Eur. J. Biochem.* 145:266-270 (1985)), several oligonucleotide primers are designed to flank the start and stop codons of the preproricin open reading frame.

The preproricin cDNA is amplified using the upstream primer Ricin-99 or Ricin-109 and the downstream primer Ricin1729C 20 with Vent DNA polymerase (New England Biolabs) using standard procedures (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)). The purified PCR fragment encoding the preproricin cDNA is then ligated into an Eco RI-digested pBluescript II SK plasmid (Stratagene), and is 25 used to transform competent XL1-Blue cells (Stratagene). The cloned PCR product containing the putative preproricin gene is confirmed by DNA sequencing of the entire cDNA clone . The sequences and location of oligonucleotide primers used for sequencing are shown in Table 1.

30 The preproricin cDNA clone is subjected to site directed mutagenesis in order to generate a series of variants differing only in the sequence between the A and B chains (linker region). The wild-type

- 31 -

preproricin linker region is replaced with the heterogenous linker sequences that are cleaved by the various disease-specific proteases as shown in Figures 21, 26, 27, 28, and Part D of Figures 30-47. Linker identification as used herein in connection with the sequences provided 5 in these figures have been assigned the sequence ID numbers as discussed below.

The linker regions of the variants encode a cleavage recognition sequence for a disease-specific protease associated with for example, cancer, viruses, parasites, or fungii. The mutagenesis and 10 cloning strategy used to generate the disease-specific protease-sensitive linker variants are summarized in Part A of Figures 2-20, and Part A of Figures 22-25. The first step involves a DNA amplification using a set of mutagenic primers in combination with the two flanking primers Richin-99Eco or Ricin-109Eco and Ricin1729C Pst I. Restriction digested 15 PCR fragments are gel purified and then ligated with PBluescript SK which has been digested with Eco RI and Pst I. Ligation reactions are used to transform competent XL1-Blue cells (Stratagene). Recombinant clones are identified by restriction digests of plasmid miniprep DNA and the mutant linker sequences are confirmed by DNA sequencing. 20 With respect to the nucleotide sequences and amino acid sequences prepared as a result of the implementation of this strategy the following sequences have been assigned the sequence ID numbers as indicated.

SEQ ID NO. 1 is used herein in connection with the DNA sequence of the baculovirus transfer vector, pVL1393.

25 The nucleotide sequence of Cathepsin B linker regions of pAP-213 are referred to herein as SEQ ID NO. 2.

The nucleotide sequence of Cathepsin B linker regions of pAP-214 are referred to herein as SEQ ID NO. 3.

30 The nucleotide sequence of MMP-3 linker regions of pAP-215 are referred to herein as SEQ ID NO. 4.

The DNA sequence of the pAP-216 insert containing ricin and the MMP-3 linker are referred to herein as SEQ ID NO. 5.

The nucleotide sequence of MMP-7 linker regions of pAP-217 are referred to herein as SEQ ID NO. 6.

The DNA sequence of the pAP-218 insert containing ricin and the MMP-7 linker are referred to herein as SEQ ID NO. 7.

5 The nucleotide sequence of MMP-9 linker regions of pAP-219 are referred to herein as SEQ ID NO. 8.

The DNA sequence of the pAP-220 insert containing ricin and the MMP-9 are referred to herein as SEQ ID NO. 9.

10 The nucleotide sequence of thermolysin-like MMP linker regions of pAP-221 are referred to herein as SEQ ID NO. 10.

The DNA sequence of of pAP-222 insert containing ricin and the thermolysin-like MMP linker are referred to herein as SEQ ID NO. 11.

15 The nucleotide sequence of Plasmodium falciparum-A linker regions of pAP-223 are referred to herein as SEQ ID NO. 12.

The DNA sequence of the pAP-224 insert containing ricin and the Plasmodium falciparum-A linker are referred to herein as SEQ ID NO. 13.

20 The nucleotide sequence of Plasmodium falciparum-B linker regions of pAP-225 are referred to herein as SEQ ID NO. 14.

The DNA sequence of the pAP-226 insert containing ricin and the Plasmodium falciparum-B linker are referred to herein as SEQ ID NO. 15.

25 The nucleotide sequence of Plasmodium falciparum-C linker regions of pAP-227 are referred to herein as SEQ ID NO. 16.

The DNA sequence of the pAP-228 insert containing ricin and the Plasmodium falciparum-C linker are referred to herein as SEQ ID NO. 17.

30 The nucleotide sequence of the the Plasmodium falciparum-D linker regions of pAP-229 is referred to herein as SEQ ID NO. 18.

The DNA sequence of the pAP-230 insert containing ricin and the Plasmodium falciparum-D linker is referred to herein as SEQ ID NO. 19.

5 The nucleotide sequence of the Plasmodium falciparum-E linker regions of pAP-231 is referred to herein as SEQ ID NO. 20.

The DNA sequence of the pAP-232 insert containing ricin and the Plasmodium falciparum-E linker is referred to herein as SEQ ID NO. 21.

10 The nucleotide sequence of the HSV-A linker regions of pAP-233 is referred to herein as SEQ ID NO. 22.

The DNA sequence of the pAP-234 insert containing ricin and the HSV-A linker is referred to herein as SEQ ID NO. 23.

The nucleotide sequence of the HSV-B linker regions of pAP-235 is referred to herein as SEQ ID NO. 24.

15 The DNA sequence of the pAP-236 insert containing ricin and the HSV-B linker is referred to herein as SEQ ID NO. 25.

The nucleotide sequence of the VZV-A linker regions of pAP-237 are referred to herein as SEQ ID NO. 26.

20 The DNA sequence of the pAP-238 insert containing ricin and the VZV-A linker are referred to herein as SEQ ID NO. 27.

The nucleotide sequence of the VZV-B linker regions of PAP-239 is referred to herein as SEQ ID NO. 28.

The DNA sequence of the pAP-240 insert containing ricin and the VZV-B linker is referred to herein as SEQ ID NO. 29.

25 The nucleotide sequence of the EBV-A linker regions of pAP-241 is referred to herein as SEQ ID NO. 30.

The DNA sequence of the pAP-242 insert containing ricin and the EBV-A linker is referred to herein as SEQ ID NO. 31.

30 The nucleotide sequence of the EBV-B linker regions of pAP-243 is referred to herein as SEQ ID NO. 32.

The DNA sequence of the pAP-244 insert containing ricin and the EBV-B linker is referred to herein as SEQ ID NO. 33.

- 34 -

The nucleotide sequence of the CMV-A linker regions of pAP-245 is referred to herein as SEQ ID NO. 34.

The DNA sequence of the pAP-246 insert containing ricin and the CMV-A linker is referred to herein as SEQ ID NO. 35.

5 The nucleotide sequence of the CMV-B linker regions of pAP-247 is referred to herein as SEQ ID NO. 36.

The DNA sequence of the pAP-248 insert containing ricin and the CMV-B linker is referred to herein as SEQ ID NO. 37.

10 The nucleotide sequence of the HHV-6 linker regions of pAP-249 is referred to herein as SEQ ID NO. 38.

The DNA sequence of the pAP-250 insert containing ricin and the HHV-6 linker is referred to herein as SEQ ID NO. 39.

15 The amino acid sequences of the cancer protease-sensitive amino acid linkers contained in the following pAP proteins have the sequence ID numbers as indicated: pAP-213 and pAP-214 (SEQ ID NO. 40); pAP-215 and pAP-216 (SEQ ID NO. 41); pAP-217 and pAP-218; (SEQ ID NO. 42); pAP-219 and pAP-220 (SEQ ID NO. 43); and pAP-221 and pAP-222 (SEQ ID NO. 44).

20 The amino acid sequences of the following cancer protease-sensitive linkers are referred to herein with the corresponding sequence ID numbers: pAP-241 and pAP-242 (SEQ ID NO. 45); and pAP-243 and pAP-244 (SEQ ID NO. 46).

The nucleotide sequence of the ILV linker regions of pAP-253 is referred to herein as SEQ ID NO. 47.

25 The DNA sequence of the pAP-254 insert containing ricin and the ILV linker is referred to herein as SEQ ID NO. 48.

The nucleotide sequence of the HAV-A linker regions of pAP-257 is referred to herein as SEQ ID NO. 49.

30 The DNA sequence of the pAP-258 insert containing ricin and HAV-A linker is referred to herein as SEQ ID NO. 50.

The nucleotide sequence of the HAV-B linker regions of pAP-255 is referred to herein as SEQ ID NO. 51.

The DNA sequence of the pAP-256 insert containing ricin and the HAV-B linker is referred to herein as SEQ ID NO. 52.

The nucleotide sequence of the CAN linker regions of pAP-259 is referred to herein as SEQ ID NO. 53.

5 The DNA sequence of the pAP-260 insert containing ricin and the CAN linker is referred to herein as SEQ ID NO. 54.

The amino acid sequences of Plasmodium falciparum protease-sensitive linkers are referred to herein by the sequence ID numbers as follows: pAP-223 and pAP-224 (SEQ ID NO 55); pAP-225 and 10 pAP-226 (SEQ ID NO 56); pAP-227 and pAP-228 (SEQ ID NO 57); pAP-229 and pAP-230 (SEQ ID NO 58); and pAP-231 and pAP-232 (SEQ ID NO 59) (see Figure 26).

15 The amino acid sequences of the viral protease-sensitive linkers which follow are referred to herein by the sequence ID numbers indicated: pAP-233 and pAP 234 (SEQ ID NO 60); pAP-235 and pAP-236 (SEQ ID NO 61); and pAP-249 and pAP-250 (SEQ ID NO 62) (see Figure 27).

20 The amino acid sequences of the viral protease-sensitive linkers which follow are referred to herein by the sequence ID numbers indicated: pAP-245 and pAP-246 (SEQ ID NO 63); and pAP-247 and pAP-248 (SEQ ID NO 64) (see Figure 27).

The amino acid sequences of the viral protease-sensitive linkers which follow are referred to herein by the sequence ID numbers indicated: pAP-237 and pAP-238 (SEQ ID NO 65); and pAP-239 and pAP-25 25 240 (SEQ ID NO 66); pAP-253 and pAP-254 (SEQ ID NO 67); pAP-255 and pAP-256 (SEQ ID NO 68); and pAP-257 and pAP-258 (SEQ ID NO 69) (see Figure 27).

30 The amino acid sequences of the *Candida* aspartic protease-sensitive linkers are referred to herein by the sequence ID numbers indicated: pAP-259 and pAP-260 (SEQ ID NO 70); pAP-261 and pAP-262 (SEQ ID NO 71); and pAP-263 and pAP-264 (SEQ ID NO 72).

- 36 -

An alternative mutagenesis and cloning strategy that can be used to generate the disease-specific protease-sensitive linker variants is summarized in Figure 29. The first step of this method involves a DNA amplification using a set of mutagenic primers in combination with the two flanking primers Ricin-109Eco and Ricin1729Pst. Restriction digested PCR fragments (Eco RI and Pst I) are gel purified. Preproricin variants produced from this method can be subcloned directly into the baculovirus transfer vector digested with Eco RI and Pst I and intermediate ligation steps involving pBluescript SK and pSB2 are circumvented. The cloning strategies used to generate disease-specific protease-sensitive linker variants are summarized in Part A of Figures 30 to 47. With respect to the nucleotide sequences and amino acid sequences prepared as a result of the implementation of this strategy the following sequences have been assigned the sequence ID numbers as indicated.

The nucleotide sequence of the HCV-A linker region of pAP-262 is referred to herein as SEQ ID NO. 73.

The DNA sequence of the pAP-262 insert is referred to herein as SEQ ID NO. 74.

The amino acid sequence of the mutant preproricin linker region for HCV-A, pAP-262, is referred to herein as SEQ ID NO. 75.

The nucleotide sequence of the HCV-B linker region of pAP-264 is referred to herein as SEQ ID NO. 76.

The DNA sequence of the pAP-264 insert is referred to herein as SEQ ID NO. 77.

The amino acid sequence of the mutant preproricin linker region for HCV-B, pAP-264, is referred to herein as SEQ ID NO. 78.

The nucleotide sequence of the HCV-C linker region of pAP-266 is referred to herein as SEQ ID NO. 79.

The DNA sequence of the pAP-266 insert is referred to herein as SEQ ID NO. 80.

The amino acid sequence of the mutant prororicin linker region for HCV-C, pAP-266, is referred to herein as SEQ ID NO. 81.

The nucleotide sequence of the HCV-D linker region of pAP-268 is referred to herein as SEQ ID NO. 82.

The DNA sequence of the pAP-268 insert is referred to herein as SEQ ID NO. 83.

10 The amino acid sequence of the mutant prororicin linker region for HCV-D , pAP-268, is referred to herein as SEQ ID NO. 84.

The nucleotide sequence of the MMP-2 linker region of pAP-270 is referred to herein as SEQ ID NO. 85.

15 The DNA sequence of the pAP-270 insert is referred to herein as SEQ ID NO. 86.

The amino acid sequence of the mutant prororicin linker region for MMP-2, pAP-270, is referred to herein as SEQ ID NO. 87.

20 The nucleotide acid sequence of the Cathepsin B (Site 2) linker region of pAP-272 is referred to herein as SEQ ID NO. 88.

The DNA sequence of the pAP-272 insert is referred to herein as SEQ ID NO. 89.

25 The amino acid sequence of the mutant prororicin linker region for Cathepsin B (Site 2), pAP-272, is referred to herein as SEQ ID NO. 90.

The nucleotide sequence of the Cathepsin L linker region of pAP-274 is referred to herein as SEQ ID NO. 91.

30 The DNA sequence of the pAP-274 insert is referred to herein as SEQ ID NO. 92.

- 38 -

The amino acid sequence of the mutant preproricin linker region of Cathepsin L, pAP-274, is referred to herein as SEQ ID NO. 93.

5 The nucleotide sequence of Cathepsin D linker region of pAP-276 is referred to herein as SEQ ID NO. 94.

The DNA sequence of the pAP-276 insert is referred to herein as SEQ ID NO. 95.

10 The amino acid sequence of the mutant preproricin linker region for Cathepsin D, pAP-276, is referred to herein as SEQ ID NO. 96.

The nucleotide sequence of the MMP-1 linker region of pAP-278 is referred to herein as SEQ ID NO. 97.

15 The DNA sequence of the pAP-278 insert is referred to herein as SEQ ID NO. 98.

The amino acid sequence of the mutant preproricin linker region for MMP-1, pAP-278, is referred to herein as SEQ ID NO. 99.

20 The nucleotide sequence of the Urokinase-Type Plasminogen Activator linker region of pAP-280 is referred to herein as SEQ ID NO. 100.

The DNA sequence of the pAP-280 insert is referred to herein as SEQ ID NO. 101.

25 The amino acid sequence of the mutant preproricin linker region for Urokinase-Type Plasminogen Activator, pAP-280, is referred to herein as SEQ ID NO. 102.

The nucleotide sequence of MT-MMP linker region of pAP-282 is referred to herein as SEQ ID NO. 103.

30 The DNA sequence of the pAP-282 insert is referred to herein as SEQ ID NO. 104.

The amino acid sequence of the mutant preproricin linker region for MT-MMP, pAP-282, is referred to herein as SEQ ID NO. 105.

- 39 -

The nucleotide sequence of the MMP-11 linker region of pAP-284 is referred to herein as SEQ ID NO. 106.

The DNA sequence of the pAP-284 insert is referred to herein as SEQ ID NO. 107.

5 The amino acid sequence of the mutant prorocin linker region for MMP-11, pAP-284, is referred to herein as SEQ ID NO. 108.

The nucleotide sequence of the MMP-13 linker region of pAP-286 is referred to herein as SEQ ID NO. 109.

10 The DNA sequence of the pAP-286 insert is referred to herein as SEQ ID NO. 110.

The amino acid sequence of the mutant prorocin linker region for MMP-13, pAP-286, is referred to herein as SEQ ID NO. 111.

15 The nucleotide sequence of the Tissue-type Plasminogen Activator linker region of pAP-288 is referred to herein as SEQ ID NO. 112.

The DNA sequence of the pAP-288 insert is referred to herein as SEQ ID NO. 113.

20 The amino acid sequence of the mutant prorocin linker region for Tissue-type Plasminogen Activator, pAP-288, is referred to herein as SEQ ID NO. 114.

The nucleotide sequence of the human Prostate-Specific Antigen linker region of pAP-290 is referred to herein as SEQ ID NO. 115.

25 The DNA sequence of the pAP-290 insert is referred to herein as SEQ ID NO. 116.

The amino acid sequence of the mutant prorocin linker region for the human Prostate-Specific Antigen, pAP-290, is 30 referred to herein as SEQ ID NO. 117.

The nucleotide sequence of the kallikrein linker region of pAP-292 is referred to herein as SEQ ID NO. 118.

- 40 -

The DNA sequence of the pAP-292 insert is referred to herein as SEQ ID NO. 119.

The amino acid sequence of the mutant prororicin linker region for the kallikrein, pAP-292, is referred to herein as SEQ ID NO. 120.

The nucleotide sequence of the neutrophil elastase linker region of pAP-294 is referred to herein as SEQ ID NO. 121.

The DNA sequence of the pAP-294 insert is referred to herein as SEQ ID NO. 122.

The amino acid sequence of the mutant prororicin linker region for neutrophil elastase, pAP-294, is referred to herein as SEQ ID NO. 123.

The nucleotide sequence of the calpain linker region of pAP-296 is referred to herein as SEQ ID NO. 124.

The DNA sequence of the pAP-296 insert is referred to herein as SEQ ID NO. 125.

The amino acid sequence of the mutant prororicin linker region for calpain, pAP-296, is referred to herein as SEQ ID NO. 126.

The amino acid sequence of the wild type linker region is referred to herein as SEQ ID NO. 127.

The nucleic acid molecule of the invention has sequences encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker sequence containing a cleavage recognition site for a disease-specific protease. The nucleic acid may be expressed to provide a recombinant protein having an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker sequence containing a cleavage recognition site for a disease-specific protease.

The nucleic acid molecule may comprise the A and/or B chain of ricin. The ricin gene has been cloned and sequenced, and the X-ray crystal structures of the A and B chains are published (Rutenber, E., et al. *Proteins* 10:240-250 (1991); Weston et al., *Mol. Biol.* 244:410-422

(1994); Lamb and Lord, *Eur. J. Biochem.* 14:265 (1985); Halling, K., et al., *Nucleic Acids Res.* 13:8019 (1985)). It will be appreciated that the invention includes nucleic acid molecules encoding truncations of A and B chains of ricin like proteins and analogs and homologs of A and

5 B chains of ricin-like proteins and truncations thereof (i.e., ricin-like proteins), as described herein. It will further be appreciated that variant forms of the nucleic acid molecules of the invention which arise by alternative splicing of an mRNA corresponding to a cDNA of the invention are encompassed by the invention.

10 Another aspect of the invention provides a nucleotide sequence which hybridizes under high stringency conditions to a nucleotide sequence encoding the A and/or B chains of a ricin-like protein. Appropriate stringency conditions which promote DNA hybridization are known to those skilled in the art, or can be found in

15 Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1 6.3.6. For example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C may be employed. The stringency may be selected based on the conditions used in the wash step. By way of example, the salt concentration in the wash step

20 can be selected from a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be at high stringency conditions, at about 65°C.

The nucleic acid molecule may comprise the A and/or B chain of a ricin-like toxin. Methods for cloning ricin-like toxins are

25 known in the art and are described, for example, in E.P. 466,222. Sequences encoding ricin or ricin-like A and B chains may be obtained by selective amplification of a coding region, using sets of degenerative primers or probes for selectively amplifying the coding region in a genomic or cDNA library. Appropriate primers may be selected from

30 the nucleic acid sequence of A and B chains of ricin or ricin-like toxins. It is also possible to design synthetic oligonucleotide primers from the nucleotide sequences for use in PCR. Suitable primers may be selected

- 42 -

from the sequences encoding regions of ricin-like proteins which are highly conserved, as described for example in U.S. Patent No 5,101,025 and E.P. 466,222.

A nucleic acid can be amplified from cDNA or genomic DNA using these oligonucleotide primers and standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. It will be appreciated that cDNA may be prepared from mRNA, by isolating total cellular mRNA by a variety of techniques, for example, by using the guanidinium-thiocyanate extraction procedure of Chirgwin et al., *Biochemistry* 18, 5294-5299 (1979). cDNA is then synthesized from the mRNA using reverse transcriptase (for example, Moloney MLV reverse transcriptase available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase available from Seikagaku America, Inc., St. Petersburg, FL). It will be appreciated that the methods described above may be used to obtain the coding sequence from plants, bacteria or fungi, preferably plants, which produce known ricin-like proteins and also to screen for the presence of genes encoding as yet unknown ricin-like proteins.

A sequence containing a cleavage recognition site for a specific protease may be selected based on the disease or the pathogen which is to be targeted by the recombinant protein. The cleavage recognition site may be selected from sequences known to encode a cleavage recognition site for the cancer, viral or parasitic protease. Sequences encoding cleavage recognition sites may be identified by testing the expression product of the sequence for susceptibility to cleavage by the respective protease.

A sequence containing a cleavage recognition site for a viral, fungal, parasitic or cancer associated protease may be selected based on the retrovirus which is to be targeted by the recombinant protein. The cleavage recognition site may be selected from sequences known to encode a cleavage recognition site for the viral, fungal,

parasitic or cancer associated protease. Sequences encoding cleavage recognition sites may be identified by testing the expression product of the sequence for susceptibility to cleavage by a viral, fungal, parasitic or cancer associated protease. A polypeptide containing the suspected 5 cleavage recognition site may be incubated with a protease and the amount of cleavage product determined (Dilannit, 1990, *J. Biol. Chem.* 285: 17345-17354 (1990)).

The protease may be prepared by methods known in the art and used to test suspected cleavage recognition sites.

10 In one embodiment, the preparation of tumour-associated cathepsin B, its substrates and enzymatic activity assay methodology have been described by Sloane, B.F. et al. (*Proc. Natl. Acad. Sci. USA* 83:2483-2487 (1986)), Schwartz, M.K. (*Clin. Chim. Acta* 237:67-78 (1995)), and Panchal, R.G. et al. (*Nature Biotechnol.* 14:852-856 (1996)).
15 The preparation of Epstein-Barr virus protease, its substrates and enzymatic activity assay methodology have been described by Welch, A.R. (*Proc. Natl. Acad. Sci. USA* 88:10792-10796 (1991)).

In another embodiment, the preparation of *Plasmodium falciparum* proteases, their substrates and enzymatic activity assay 20 methodology have been described by Goldberg, D.E. et al. (*J. Exp. Med.* 173:961-969 (1991)), Cooper & Bujard (*Mol. Biochem. Parasitol.* 56:151-160 (1992)), Nwagwu, M. et al. (*Exp. Parasitol.* 75:399-414 (1992)), Rosenthal, P.J. et al. (*J. Clin. Invest.* 91:1052-1056 (1993)), Blackman, M.J. et al. (*Mol. Biochem. Parasitol.* 62:103-114 (1995)).

25 In a further embodiment, the preparation of proteases from human cytomegalovirus, human herpes virus, varicella zoster virus and infectious laryngotracheitis virus have been taught by Liu F. & Roizman, B. (*J. Virol.* 65:5149-5156 (1991)) and Welch, A.R. (*Proc. Natl. Acad. Sci. USA* 88:10792-10796 (1991)). In addition, their respective 30 substrates and enzymatic activity assay methodologies are also described.

In another embodiment, the preparation of hepatitis A virus protease, its substrates and enzymatic activity assay methodology have been described by Jewell, D.A. et al. (*Biochemistry* 31:7862-7869 (1992)). The preparation of poliovirus protease, its substrates and enzymatic activity assay methodology have been described by Weidner, J.R. et al. (*Arch. Biochem. Biophys.* 286:402-408 (1991)). The preparation of human rhinovirus protease, its substrates and enzymatic activity assay methodology have been described by Long, A.C. et al. (*FEBS Lett.* 258:75-78 (1989)).

In another embodiment of the invention, the preparation of proteases associated with *Candida* yeasts their substrates and enzymatic activity are contemplated, including the aspartic proteinases which have been associated specifically with numerous virulent strains of *Candida* including *Candida albican*, *Candida tropicalis*, and *Candida parapsilosis* (Abad-Zapatero, C. et al., *Protein Sci.* 5:640-652 (1996); Cutfield, S.M. et al., *Biochemistry* 35:398-410 (1995); Ruchel, R. et al, *Zentralbl. Bakteriol. Mikrobiol Hyg. I Abt. Orig. A.* 255:537-548 (1983); Remold, H. et al., *Biochim. Biophys. Acta* 167:399-406 (1968)).

The nucleic acid molecule of the invention may be prepared by site directed mutagenesis. For example, the cleavage site of a disease-specific protease may be prepared by site directed mutagenesis of the homologous linker sequence of a proricin-like toxin. Procedures for cloning proricin-like genes, encoding a linker sequence are described in EP 466,222. Site directed mutagenesis may be accomplished by DNA amplification of mutagenic primers in combination with flanking primers. Suitable procedures using the mutagenic primers are shown in Parts A and B of Figures 1-4, Figures 13-16, Figures 18-36, Figures 38-41, and Figures 50-67.

The nucleic acid molecule of the invention may also encode a fusion protein. A sequence encoding a heterologous linker sequence containing a cleavage recognition site for a disease-specific protease may be cloned from a cDNA or genomic library or chemically

synthesized based on the known sequence of such cleavage sites. The heterologous linker sequence may then be fused in frame with the sequences encoding the A and B chains of the ricin-like toxin for expression as a fusion protein. It will be appreciated that a nucleic acid
5 molecule encoding a fusion protein may contain a sequence encoding an A chain and a B chain from the same ricin-like toxin or the encoded A and B chains may be from different toxins. For example, the A chain may be derived from ricin and the B chain may be derived from abrin. A protein may also be prepared by chemical conjugation of the A and B
10 chains and linker sequence using conventional coupling agents for covalent attachment.

An isolated and purified nucleic acid molecule of the invention which is RNA can be isolated by cloning a cDNA encoding an A and B chain and a linker into an appropriate vector which allows
15 for transcription of the cDNA to produce an RNA molecule which encodes a protein of the invention. For example, a cDNA can be cloned downstream of a bacteriophage promoter, (e.g. a T7 promoter) in a vector, cDNA can be transcribed in vitro with T7 polymerase, and the resultant RNA can be isolated by standard techniques.

20 Recombinant Protein of the Invention

As previously mentioned, the invention provides novel recombinant proteins which incorporate the A and B chains of a ricin like toxin linked by a heterologous linker sequence containing a cleavage recognition site for a disease-specific protease. It is an
25 advantage of the recombinant proteins of the invention that they are non-toxic until the A chain is liberated from the B chain by specific cleavage of the linker by the target protease.

Thus the protein may be used to specifically target cancer cells or cells infected with a virus or parasite in the absence of additional
30 specific cell-binding components to target infected cells. It is a further advantage that the disease-specific protease cleaves the heterologous linker intracellularly thereby releasing the toxic A chain directly into

- 46 -

the cytoplasm of the cancer cell or infected cell. As a result, said cells are specifically targeted and non-infected normal cells are not directly exposed to the activated free A chain.

Ricin is a plant derived ribosome inhibiting protein
5 which blocks protein synthesis in eukaryotic cells. Ricin may be derived from the seeds of *Ricinus communis* (castor oil plant). The ricin toxin is a glycosylated heterodimer with A and B chain molecular masses of 30,625 Da and 31,431 Da respectively. The A chain of ricin has an N-glycosidase activity and catalyzes the excision of a specific adenine
10 residue from the 28S rRNA of eukaryotic ribosomes (Endo, Y; & Tsurugi, K. J. Biol. Chem. 262:8128 (1987)). The B chain of ricin, although not toxic in itself, promotes the toxicity of the A chain by binding to galactose residues on the surface of eukaryotic cells and stimulating receptor-mediated endocytosis of the toxin molecule
15 (Simmons et al., Biol. Chem. 261:7912 (1986)).

All protein toxins are initially produced in an inactive, precursor form. Ricin is initially produced as a single polypeptide (preproricin) with a 35 amino acid N-terminal presequence and 12 amino acid linker between the A and B chains. The pre-sequence is
20 removed during translocation of the ricin precursor into the endoplasmic reticulum (Lord, J.M., Eur. J. Biochem. 146:403-409 (1985) and Lord, J.M., Eur. J. Biochem. 146:411-416 (1985)). The proricin is then translocated into specialized organelles called protein bodies where a plant protease cleaves the protein at a linker region between the A and
25 B chains (Lord, J.M. et al., FASAB Journal 8:201-208 (1994)). The two chains, however, remain covalently attached by an interchain disulfide bond (cysteine 259 in the A chain to cysteine 4 in the B chain) and mature disulfide linked ricin is stored in protein bodies inside plant cells. The A chain is inactive in the proricin (O'Hare, M., et al., FEBS
30 Lett. 273:200-204 (1990)) and it is inactive in the disulfide-linked mature ricin (Richardson, P.T. et al., FEBS Lett. 255:15-20 (1989)). The ribosomes of the castor bean plant are themselves susceptible to inactivation by

ricin A chain; however, as there is no cell surface galactose to permit B chain recognition the A chain cannot re-enter the cell.

Ricin-like proteins include, but are not limited to, bacterial, fungal and plant toxins which have A and B chains and 5 inactivate ribosomes and inhibit protein synthesis. The A chain is an active polypeptide subunit which is responsible for the pharmacologic effect of the toxin. In most cases the active component of the A chain is an enzyme. The B chain is responsible for binding the toxin to the cell surface and is thought to facilitate entry of the A chain into the cell 10 cytoplasm. The A and B chains in the mature toxins are linked by disulfide bonds. The toxins most similar in structure to ricin are plant toxins which have one A chain and one B chain. Examples of such toxins include abrin which may be isolated from the seeds of *Abrus precatorius* and modeccin.

15 Ricin-like bacterial proteins include diphtheria toxin, which is produced by *Corynebacterium diphtheriae*, *Pseudomonas enterotoxin A* and cholera toxin. It will be appreciated that the term ricin-like toxins is also intended to include the A chain of those toxins which have only an A chain. The recombinant proteins of the 20 invention could include the A chain of these toxins conjugated to, or expressed as, a recombinant protein with the B chain of another toxin. Examples of plant toxins having only an A chain include trichosanthin, MMC and pokeweed antiviral proteins, dianthin 30, dianthin 32, crotin II, curcin II and wheat germ inhibitor. Examples of fungal toxins 25 having only an A chain include alpha-sarcin, restrictocin, mitogillin, enomycin, phenomycin. Examples of bacterial toxins having only an A chain include cytotoxin from *Shigella dysenteriae* and related Shiga-like toxins. Recombinant trichosanthin and the coding sequence thereof is disclosed in U.S. Patents 5,101,025 and 5,128,460.

30 In addition to the entire A or B chains of a ricin-like toxin, it will be appreciated that the recombinant protein of the invention may contain only that portion of the A chain which is

necessary for exerting its cytotoxic effect. For example, the first 30 amino acids of the ricin A chain may be removed resulting in a truncated A chain which retains toxic activity. The truncated ricin or ricin-like A chain may be prepared by expression of a truncated gene or by

5 proteolytic degradation, for example with Nagarse (Funmatsu et al., *Jap. J. Med. Sci. Biol.* 23:264-267 (1970)). Similarly, the recombinant protein of the invention may contain only that portion of the B chain necessary for galactose recognition, cell binding and transport into the cell cytoplasm. Truncated B chains are described for example in E.P.

10 145,111. The A and B chains may be glycosylated or non-glycosylated. Glycosylated A and B chains may be obtained by expression in the appropriate host cell capable of glycosylation. Non-glycosylated chains may be obtained by expression in nonglycosylating host cells or by treatment to remove or destroy the carbohydrate moieties.

15 The proteins of the invention may be prepared using recombinant DNA methods. Accordingly, the nucleic acid molecules of the present invention may be incorporated in a known manner into an appropriate expression vector which ensures good expression of the protein. Possible expression vectors include but are not limited to

20 cosmids, plasmids, or modified viruses (e.g. replication defective retroviruses, adenoviruses and adeno-associated viruses), so long as the vector is compatible with the host cell used. The expression vectors are "suitable for transformation of a host cell", which means that the expression vectors contain a nucleic acid molecule of the invention and

25 regulatory sequences selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid molecule. Operatively linked is intended to mean that the nucleic acid is linked to regulatory sequences in a manner which allows expression of the nucleic acid.

30 The invention therefore contemplates a recombinant expression vector of the invention containing a nucleic acid molecule of the invention, or a fragment thereof, and the necessary regulatory

sequences for the transcription and translation of the inserted protein-sequence.

Suitable regulatory sequences may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes (For example, see the regulatory sequences described in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Selection of appropriate regulatory sequences is dependent on the host cell chosen as discussed below, and may be readily accomplished by one of ordinary skill in the art. Examples of such regulatory sequences include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the host cell chosen and the vector employed, other sequences, such as an origin of replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription may be incorporated into the expression vector. It will also be appreciated that the necessary regulatory sequences may be supplied by the native A and B chains and/or its flanking regions.

The recombinant expression vectors of the invention may also contain a selectable marker gene which facilitates the selection of host cells transformed or transfected with a recombinant molecule of the invention. Examples of selectable marker genes are genes encoding a protein such as G418 and hygromycin which confer resistance to certain drugs, β -galactosidase, chloramphenicol acetyltransferase, firefly luciferase, or an immunoglobulin or portion thereof such as the Fc portion of an immunoglobulin preferably IgG. Transcription of the selectable marker gene is monitored by changes in the concentration of the selectable marker protein such as β -galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. If the selectable marker gene encodes a protein conferring antibiotic resistance such as neomycin resistance transformant cells can be selected with G418. Cells that have

- 50 -

incorporated the selectable marker gene will survive, while the other cells die. This makes it possible to visualize and assay for expression of recombinant expression vectors of the invention and in particular to determine the effect of a mutation on expression and phenotype. It will
5 be appreciated that selectable markers can be introduced on a separate vector from the nucleic acid of interest.

The recombinant expression vectors may also contain genes which encode a fusion moiety which provides increased expression of the recombinant protein; increased solubility of the
10 recombinant protein; and aid in the purification of the target recombinant protein by acting as a ligand in affinity purification. For example, a proteolytic cleavage site may be added to the target recombinant protein to allow separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein.
15 Typical fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the recombinant protein.

20 Recombinant expression vectors can be introduced into host cells to produce a transformant host cell. The term "transformant host cell" is intended to include prokaryotic and eukaryotic cells which have been transformed or transfected with a recombinant expression vector of the invention. The terms "transformed with", "transfected
25 with", "transformation" and "transfection" are intended to encompass introduction of nucleic acid (e.g. a vector) into a cell by one of many possible techniques known in the art. Prokaryotic cells can be transformed with nucleic acid by, for example, electroporation or calcium-chloride mediated transformation. Nucleic acid can be
30 introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran mediated transfection, lipofectin, electroporation or microinjection.

Suitable methods for transforming and transfecting host cells can be found in Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks.

5 Suitable host cells include a wide variety of prokaryotic and eukaryotic host cells. For example, the proteins of the invention may be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus), yeast cells or mammalian cells. Other suitable host cells can be found in Goeddel, *Gene Expression Technology: Methods in*
10 *Enzymology* 185, Academic Press, San Diego, CA (1991).

More particularly, bacterial host cells suitable for carrying out the present invention include *E. coli*, *B. subtilis*, *Salmonella typhimurium*, and various species within the genus' *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, as well as many other bacterial
15 species well known to one of ordinary skill in the art. Suitable bacterial expression vectors preferably comprise a promoter which functions in the host cell, one or more selectable phenotypic markers, and a bacterial origin of replication. Representative promoters include the β -lactamase (penicillinase) and lactose promoter system (see Chang et al., *Nature* 275:615 (1978)), the trp promoter (Nichols and Yanofsky, *Meth in Enzymology* 101:155, (1983) and the tac promoter (Russell et al., *Gene* 20: 231, (1982)). Representative selectable markers include various antibiotic resistance markers such as the kanamycin or ampicillin resistance genes. Suitable expression vectors include but are not limited
20 to bacteriophages such as lambda derivatives or plasmids such as pBR322 (Bolivar et al., *Gene* 2:9S, (1977)), the pUC plasmids pUC18, pUC19, pUC118, pUC119 (see Messing, *Meth in Enzymology* 101:20-77, 1983 and Vieira and Messing, *Gene* 19:259-268 (1982)), and pNH8A, pNH16a, pNH18a, and Bluescript M13 (Stratagene, La Jolla, Calif.).
25
30 Typical fusion expression vectors which may be used are discussed above, e.g. pGEX (Amrad Corp., Melbourne, Australia), pMAL (New

- 52 -

England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ).

Examples of inducible non-fusion expression vectors include pTrc (Amann et al., *Gene* 69:301-315 (1988)) and pET 11d (Studier et al., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press,

5 San Diego, California, 60-89 (1990)).

Yeast and fungi host cells suitable for carrying out the present invention include, but are not limited to *Saccharomyces cerevisiae*, the genera *Pichia* or *Kluyveromyces* and various species of the genus *Aspergillus*. Examples of vectors for expression in yeast *S.*

10 *cerevisiae* include pYEPSec1 (Baldari. et al., *Embo J.* 6:229-234 (1987)), pMFa (Kurjan and Herskowitz, *Cell* 30:933-943 (1982)), pJRY88 (Schultz et al., *Gene* 54:113-123 (1987)), and pYES2 (Invitrogen Corporation, San Diego, CA). Protocols for the transformation of yeast and fungi are well known to those of ordinary skill in the art.(see Hinnen et al., *Proc. Natl. Acad. Sci. USA* 75:1929 (1978); Itoh et al., *J. Bacteriology* 153:163 (1983), and Cullen et al. (*Bio/Technology* 5:369 (1987)).

Mammalian cells suitable for carrying out the present invention include, among others: COS (e.g., ATCC No. CRL 1650 or 1651), BHK (e.g. ATCC No. CRL 6281), CHO (ATCC No. CCL 61), HeLa 20 (e.g., ATCC No. CCL 2), 293 (ATCC No. 1573) and NS-1 cells. Suitable expression vectors for directing expression in mammalian cells generally include a promoter (e.g., derived from viral material such as polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40), as well as other transcriptional and translational control sequences. Examples 25 of mammalian expression vectors include pCDM8 (Seed, B., *Nature* 329:840 (1987)) and pMT2PC (Kaufman et al., *EMBO J.* 6:187-195 (1987)).

Given the teachings provided herein, promoters, terminators, and methods for introducing expression vectors of an appropriate type into plant, avian, and insect cells may also be readily 30 accomplished. For example, within one embodiment, the proteins of the invention may be expressed from plant cells (see Sinkar et al., *J. Biosci* (Bangalore) 11:47-58 (1987), which reviews the use of

Agrobacterium rhizogenes vectors; see also Zambryski et al., *Genetic Engineering, Principles and Methods*, Hollaender and Setlow (eds.), Vol. VI, pp. 253-278, Plenum Press, New York (1984), which describes the use of expression vectors for plant cells, including, among others,
5 pAS2022, pAS2023, and pAS2034).

Insect cells suitable for carrying out the present invention include cells and cell lines from *Bombyx*, *Trichoplusia* or *Spodotera* species. Baculovirus vectors available for expression of proteins in cultured insect cells (SF 9 cells) include the pAc series (Smith et al., *Mol.*
10 *Cell Biol.* 3:2156-2165 (1983)) and the pVL series (Lucklow, V.A., and Summers, M.D., *Virology* 170:31-39 (1989)). Some baculovirus-insect cell expression systems suitable for expression of the recombinant proteins of the invention are described in PCT/US/02442.

Alternatively, the proteins of the invention may also be
15 expressed in non-human transgenic animals such as, rats, rabbits, sheep and pigs (Hammer et al. *Nature* 315:680-683 (1985); Palmiter et al. *Science* 222:809-814 (1983); Brinster et al. *Proc. Natl. Acad. Sci. USA* 82:4438-4442 (1985); Palmiter and Brinster *Cell* 41:343-345 (1985) and U.S. Patent No. 4,736,866).

20 The proteins of the invention may also be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, *J. Am. Chem. Assoc.* 85:2149-2154 (1964)) or synthesis in homogenous solution (Houbenweyl, *Methods of Organic Chemistry*, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart (1987)).

The present invention also provides proteins comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a
30 disease-specific protease. Such a protein could be prepared other than by recombinant means, for example by chemical synthesis or by conjugation of A and B chains and a linker sequence isolated and

- 54 -

purified from their natural plant, fungal or bacterial source. Such A and B chains could be prepared having the glycosylation pattern of the native ricin-like toxin.

N-terminal or C-terminal fusion proteins comprising the 5 protein of the invention conjugated with other molecules, such as proteins may be prepared by fusing, through recombinant techniques. The resultant fusion proteins contain a protein of the invention fused to the selected protein or marker protein as described herein. The recombinant protein of the invention may also be conjugated to other 10 proteins by known techniques. For example, the proteins may be coupled using heterobifunctional thiol-containing linkers as described in WO 90/10457, N-succinimidyl-3-(2-pyridyldithio-propionate) or N-succinimidyl-5 thioacetate. Examples of proteins which may be used to prepare fusion proteins or conjugates include cell binding proteins 15 such as immunoglobulins, hormones, growth factors, lectins, insulin, low density lipoprotein, glucagon, endorphins, transferrin, bombesin, asialoglycoprotein glutathione-S-transferase (GST), hemagglutinin (HA), and truncated myc.

Utility of the Nucleic Acid Molecules and Proteins of the Invention

The proteins of the invention may be used to specifically 20 inhibit or destroy mammalian cells affected by a disease or infection which have associated with such cells a specific protease, i.e., disease-specific, for example cancer cells or cells infected with a virus, fungus or parasite, all of which are encompassed within the term "disease-specific." 25 It is an advantage of the recombinant proteins of the invention that they have specificity for said cells without the need for a cell binding component. The ricin-like B chain of the recombinant proteins recognize galactose moieties on the cell surface and ensure that the protein is taken up by the diseased cell and released into the cytoplasm. 30 When the protein is internalized into a non-infected cell, cleavage of the heterologous linker would not occur in the absence of the disease-specific protease and the A chain will remain inactive bound to the B

chain. Conversely, when the protein is internalized into a diseased cell, the disease-specific protease will cleave the cleavage recognition site in the linker thereby releasing the toxic A chain.

The specificity of a recombinant protein of the invention

- 5 may be tested by treating the protein with the disease-specific protease which is thought to be specific for the cleavage recognition site of the linker and assaying for cleavage products. Disease-specific proteases may be isolated from cancer cells or infected cells, or they may be prepared recombinantly, for example following the procedures in
- 10 Darket et al. (*J. Biol. Chem.* 254:2307-2312 (1988)). The cleavage products may be identified for example based on size, antigenicity or activity. The toxicity of the recombinant protein may be investigated by subjecting the cleavage products to an *in vitro* translation assay in cell lysates, for example using Brome Mosaic Virus mRNA as a template.
- 15 Toxicity of the cleavage products may be determined using a ribosomal inactivation assay (Westby et al., *Bioconjugate Chem.* 3:377-382 (1992)). The effect of the cleavage products on protein synthesis may be measured in standardized assays of *in vitro* translation utilizing partially defined cell free systems composed for example of a
- 20 reticulocyte lysate preparation as a source of ribosomes and various essential cofactors, such as mRNA template and amino acids. Use of radiolabelled amino acids in the mixture allows quantitation of incorporation of free amino acid precursors into trichloroacetic acid precipitable proteins. Rabbit reticulocyte lysates may be conveniently used (O'Hare, *FEBS Lett.* 273:200-204 (1990)).
- 25

The ability of the recombinant proteins of the invention to selectively inhibit or destroy animal cancer cells or cells infected with a virus or parasite may be readily tested *in vitro* using animal cancer cell lines or cell cultures infected with the virus or parasite of interest.

- 30 The selective inhibitory effect of the recombinant proteins of the invention may be determined, for example, by demonstrating the selective inhibition of viral antigen expression in infected mammalian

cells, the selective inhibition of general mRNA translation and protein synthesis in diseased cells, or selective inhibition of cellular proliferation in cancer cells or infected cells.

Toxicity may also be measured based on cell viability, for example the viability of infected and non-infected cell cultures exposed to the recombinant protein may be compared. Cell viability may be assessed by known techniques, such as trypan blue exclusion assays.

In another example, a number of models may be used to test the cytotoxicity of recombinant proteins having a heterologous linker sequence containing a cleavage recognition site for a cancer-associated matrix metalloprotease. Thompson, E.W. et al. (*Breast Cancer Res. Treatment* 31:357-370 (1994)) has described a model for the determination of invasiveness of human breast cancer cells *in vitro* by measuring tumour cell-mediated proteolysis of extracellular matrix and tumour cell invasion of reconstituted basement membrane (collagen, laminin, fibronectin, Matrigel or gelatin). Other applicable cancer cell models include cultured ovarian adenocarcinoma cells (Young, T.N. et al. *Gynecol. Oncol.* 62:89-99 (1996); Moore, D.H. et al. *Gynecol. Oncol.* 65:78-82 (1997)), human follicular thyroid cancer cells (Demeure, M.J. et al., *World J. Surg.* 16:770-776 (1992)), human melanoma (A-2058) and fibrosarcoma (HT-1080) cell lines (Mackay, A.R. et al. *Lab. Invest.* 70:781-783 (1994)), and lung squamous (HS-24) and adenocarcinoma (SB-3) cell lines (Spiess, E. et al. *J. Histochem. Cytochem.* 42:917-929 (1994)). An *in vivo* test system involving the implantation of tumours and measurement of tumour growth and metastasis in athymic nude mice has also been described (Thompson, E.W. et al., *Breast Cancer Res. Treatment* 31:357-370 (1994); Shi, Y.E. et al., *Cancer Res.* 53:1409-1415 (1993)).

A further model may be used to test the cytotoxicity of recombinant proteins having a heterologous linker sequence containing a cleavage recognition site for a cancer-associated Cathepsin

B protease is provided in human glioma (Mikkelsen, T. et al. *J. Neurosurge*, 83:285-290 (1995)).

Similarly, the cytotoxicity of recombinant proteins having a heterologous linker sequence containing a cleavage recognition site 5 for a malarial protease may be tested by a Plasmodium invasion assay using human erythrocytes infected with mature-stage merozoite parasites as described by McPherson, R.A. et al. (*Mol. Biochem. Parasitol.* 62:233-242 (1993)). Alternatively, in vitro cultures of human hepatic parenchymal cells may be used to evaluate schizont infectivity and 10 Plasmodium merozoite generation.

With respect to models of viral infection and replication, suitable animal cells which can be cultured *in vitro* and which are capable of maintaining viral replication can be used as hosts. The toxicity of the recombinant protein for infected and non-infected 15 cultures may then be compared. The ability of the recombinant protein of the invention to inhibit the expression of these viral antigens may be an important indicator of the ability of the protein to inhibit viral replication. Levels of these antigens may be measured in assays using labelled antibodies having specificity for the antigens. Inhibition of 20 viral antigen expression has been correlated with inhibition of viral replication (U.S. Patent No. 4,869,903). Toxicity may also be assessed based on a decrease in protein synthesis in target cells, which may be measured by known techniques, such as incorporation of labelled amino acids, such as [3H] leucine (O'Hare et al., *FEBS Lett.* 273:200-204 25 (1990)). Infected cells may also be pulsed with radiolabelled thymidine and incorporation of the radioactive label into cellular DNA may be taken as a measure of cellular proliferation. Toxicity may also be measured based on cell death or lysis, for example, the viability of 30 infected and non-infected cell cultures exposed to the recombinant protein may be compared. Cell viability may be assessed by known techniques, such as trypan blue exclusion assays.

Although the primary specificity of the proteins of the invention for diseased cells is mediated by the specific cleavage of the cleavage recognition site of the linker, it will be appreciated that specific cell binding components may optionally be conjugated to the proteins 5 of the invention. Such cell binding components may be expressed as fusion proteins with the proteins of the invention or the cell binding component may be physically or chemically coupled to the protein component. Examples of suitable cell binding components include antibodies to cancer, viral or parasitic proteins.

10 Antibodies having specificity for a cell surface protein may be prepared by conventional methods. A mammal, (e.g. a mouse, hamster, or rabbit) can be immunized with an immunogenic form of the peptide which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on a peptide include 15 conjugation to carriers or other techniques well known in the art. For example, the peptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay procedures can be used with the immunogen as antigen 20 to assess the levels of antibodies. Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera.

To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and 25 fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art, (e.g. the hybridoma technique originally developed by Kohler and Milstein (*Nature* 256:495-497 (1975)) as well as other techniques such as the human B-cell hybridoma 30 technique (Kozbor et al., *Immunol. Today* 4:72 (1983)), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., *Monoclonal Antibodies in Cancer Therapy* Allen R., Bliss,

Inc., pages 77-96 (1985)), and screening of combinatorial antibody libraries (Huse et al., *Science* 246:1275 (1989)). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the peptide and the monoclonal antibodies can be isolated.

5 The term "antibody" as used herein is intended to include fragments thereof which also specifically react with a cell surface component. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above. For example, F(ab')2 fragments can be generated by
10 treating antibody with pepsin. The resulting F(ab')2 fragment can be treated to reduce disulfide bridges to produce Fab' fragments.

Chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region are also contemplated within the scope of the
15 invention. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. Conventional methods may be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes a cell surface antigen (See, for
20 example, Morrison et al., *Proc. Natl Acad. Sci. U.S.A.* 81:6851 (1985); Takeda et al., *Nature* 314:452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., E.P. Patent No. 171,496; European Patent No. 173,494, United Kingdom Patent No. GB 2177096B). It is expected that chimeric antibodies would
25 be less immunogenic in a human subject than the corresponding non-chimeric antibody.

Monoclonal or chimeric antibodies specifically reactive against cell surface components can be further humanized by producing human constant region chimeras, in which parts of the variable
30 regions, particularly the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. Such

- 60 -

immunoglobulin molecules may be made by techniques known in the art, (e.g. Teng et al., *Proc. Natl. Acad. Sci. U.S.A.*, 80:7308-7312 (1983); Kozbor et al., *Immunology Today* 4:7279 (1983); Olsson et al., *Meth. Enzymol.*, 92:3-16 (1982), and PCT Publication WO92/06193 or EP 5 239,400). Humanized antibodies can also be commercially produced (Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.)

Specific antibodies, or antibody fragments, reactive against cell surface components may also be generated by screening expression libraries encoding immunoglobulin genes, or portions 10 thereof, expressed in bacteria with cell surface components. For example, complete Fab fragments, VH regions and FV regions can be expressed in bacteria using phage expression libraries (See for example Ward et al., *Nature* 341:544-546 (1989); Huse et al., *Science* 246:1275-1281 (1989); and McCafferty et al., *Nature* 348:552-554 (1990)). Alternatively, a 15 SCID-hu mouse, for example the model developed by Genpharm, can be used to produce antibodies, or fragments thereof.

The proteins of the invention may be formulated into pharmaceutical compositions for administration to subjects in a biologically compatible form suitable for administration *in vivo*. By 20 "biologically compatible form suitable for administration *in vivo*" is meant a form of the substance to be administered in which any toxic effects are outweighed by the therapeutic effects. The substances may be administered to living organisms including humans, and animals. Administration of a therapeutically active amount of the 25 pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of antibody 30 to elicit a desired response in the individual. Dosage regime may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be

proportionally reduced as indicated by the exigencies of the therapeutic situation.

The nucleic acid molecules of the invention may be formulated into pharmaceutical compositions for administration to subjects in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the substance to be administered in which any toxic effects are outweighed by the therapeutic effects. The substances may be administered to living organisms including humans, and animals. Administration of a therapeutically active amount of the pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of antibody to elicit a desired response in the individual. Dosage regime may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The active substance may be administered in a convenient manner such as by injection (subcutaneous, intravenous, intramuscular, etc.), oral administration, inhalation, transdermal administration (such as topical cream or ointment, etc.), or suppository applications. Depending on the route of administration, the active substance may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound.

The compositions described herein can be prepared by *per se* known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with

a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985). On this basis, the compositions include, albeit not exclusively, 5 solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

The pharmaceutical compositions may be used in 10 methods for treating animals, including mammals, preferably humans, with cancer or infected with a virus or a parasite. It is anticipated that the compositions will be particularly useful for treating patients with B-cell lymphoproliferative disease, (melanoma), mononucleosis, cytomegalic inclusion disease, malaria, herpes, shingles, hepatitis, 15 poliomyelitis, or infectious laryngotracheitis. The dosage and type of recombinant protein to be administered will depend on a variety of factors which may be readily monitored in human subjects. Such factors include the etiology and severity (grade and stage) of neoplasia, the stage of malarial infection (e.g. exoerythrocytic *vs.* erythrocytic), or 20 antigen levels associated with viral load in patient tissues or circulation.

As mentioned above, the novel recombinant toxic proteins and nucleic acid molecules of the present invention are useful in treating cancerous or infected cells wherein the cells contain a specific protease that can cleave the linker region of the recombinant toxic 25 protein. One skilled in the art can appreciate that many different recombinant toxic proteins can be prepared once a disease associated protease has been identified. For example, the novel recombinant toxic proteins and nucleic acid molecules of the invention may be used to treat CNS tumors. Muller et al. (1993) describe increased activity of 30 Insulin-type Growth Factor Binding Protein-3 (IGFBP-3) protease in the Cerebral Spinal Fluid of patients with CNS tumors. Cohen et al. (1992) claim that prostate-specific antigen (PSA) is an IGFBP-3 protease. The

pAP290 construct described above is a substrate for PSA. Conover et al. (1994) claim that cathepsin D is IGFBP-3 protease. The pAP276 described herein is a substrate for cathepsin D. Another example of a specific use of the invention is treatment of human glioma which has been shown 5 to produce cathepsin D (Mikkelsen, T. et al. *J. Neurosurgery*, 83:285-290 (1995)). The pAP 214 and 272 define herein are substrates for cathepsin B.

In addition, the novel proteins and nucleic acid molecules of the present invention may be used to treat cystic fibrosis. 10 Hansen et al. (1995) describe how CF airway disease is characterized by neutrophil-dominated chronic inflammation with an excess of uninhibited neutrophil elastase (NE). NE levels in CF sputum are 350 times higher than that found in normal sputum. The pAP294 described herein is a substrate for neutrophil elastase.

15 As well, the novel proteins and nucleic acid molecules of the present invention may also be used to treat multiple sclerosis. Bever Jr. et al. (1994) implicate cathepsin B (possibly from inflammatory cells of hematogenous origin) in the demyelination found in multiple sclerosis. pAPs 214 and 272 defined herein present substrates for 20 cathepsin B.

The term "animal" as used herein includes all members of the animal kingdom including mammals, preferably humans.

The following non-limiting examples are illustrative of the present invention:

25 **EXAMPLES**

Example 1

Cloning and Expression of Proricin Variants Activated by Disease-Specific Proteases

Isolation of total RNA

30 The preproricin gene was cloned from new foliage of the castor bean plant. Total messenger RNA was isolated according to established procedures (Sambrook et al., *Molecular Cloning: A Lab*

Manual (Cold Spring Harbour Press, Cold Spring Harbour, (1989)) and cDNA generated using reverse transcriptase.

cDNA Synthesis:

Oligonucleotides, corresponding to the extreme 5' and 3' ends of the preproricin gene were synthesized and used to PCR amplify the gene. Using the cDNA sequence for preproricin (Lamb et al., Eur. J. Biochem., 145:266-270, 1985), several oligonucleotide primers were designed to flank the start and stop codons of the preproricin open reading frame. The oligonucleotides were synthesized using an Applied Biosystems Model 392 DNA/RNA Synthesizer. First strand cDNA synthesis was primed using the oligonucleotide Ricin1729C (Table 1). Three micrograms of total RNA was used as a template for oligo Ricin1729C primed synthesis of cDNA using Superscript II Reverse Transcriptase (BRL) following the manufacturer's protocol.

15 DNA Amplification and Cloning

The first strand cDNA synthesis reaction was used as template for DNA amplification by the polymerase chain reaction (PCR). The preproricin cDNA was amplified using the upstream primer Ricin-99 and the downstream primer Ricin1729C with Vent DNA polymerase (New England Biolabs) using standard procedures (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)). Amplification was carried out in a Biometra thermal cycler (TRIO-Thermalcycler) using the following cycling parameters: denaturation 95°C for 1 min., annealing 52°C for 1 min., and extension 72°C for 2 min., (33 cycles), followed by a final extension cycle at 72°C for 10 min. The 1846bp amplified product was fractionated on an agarose gel (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)), and the DNA purified from the gel slice using Qiaex resin (Qiagen) following the manufacturer's protocol. The purified PCR fragment encoding the preproricin cDNA was then ligated (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second

Edition, (Cold Spring Harbor Laboratory Press, 1989)) into an Eco RV-digested pBluescript II SK plasmid (Stratagene), and used to transform competent XL1-Blue cells (Stratagene). Positive clones were confirmed by restriction digestion of purified plasmid DNA. Plasmid DNA was
5 extracted using a Qiaprep Spin Plasmid Miniprep Kit (Qiagen).

DNA Sequencing

The cloned PCR product containing the putative preproricin gene was confirmed by DNA sequencing of the entire cDNA clone (pAP-144). Sequencing was performed using an Applied
10 Biosystems 373A Automated DNA Sequencer, and confirmed by double-stranded dideoxy sequencing by the Sanger method using the Sequenase kit (USB). The oligonucleotide primers used for sequencing were as follows: Ricin267, Ricin486, Ricin725, Ricin937, Ricin1151, Ricini1399, Ricin1627, T3 primer
15 (5'AATTAACCCTCACTAAAGGG-3') (SEQ ID NO. 128) and T7 primer (5'GTAATACGACTCACTATAGGGC-3) (SEQ ID NO. 129). Sequence data was compiled and analyzed using PC Gene software package (intelligenetics). The sequences and location of oligonucleotide primers is shown in Table 1. The oligonucleotide primers shown in Table 1
20 have been assigned the following sequence ID numbers:
Ricin-109 is referred to herein as SEQ ID NO. 130;
Ricin-99Eco is referred to herein as SEQ ID NO. 131;
Ricin267 is referred to herein as SEQ ID NO. 132;
Ricin486 is referred to herein as SEQ ID NO. 133;
25 Ricin725 is referred to herein as SEQ ID NO. 134;
Ricin 937 is referred to herein as SEQ ID NO. 135;
Ricin 1151 is referred to herein as SEQ ID NO. 136;
Ricin 1399 is referred to herein as SEQ ID NO. 137;
Ricin 1627 is referred to herein as SEQ ID NO. 138;
30 Ricin 1729C is referred to herein as SEQ ID NO. 139; and
Ricin 1729C Xba is referred to herein as SEQ ID NO. 140.

Production and Cloning of Linker Variants

- 66 -

pAP144 cut with EcoRI was used as target for PCR pairs employing the Ricin109-Eco oligonucleotide (Ricin-109Eco primer: 5-GGAGGAATCCGGAGATGAAACCGGGAGGAAATACTATTGTAAT-3 (SEQ ID No. 141)) and a mutagenic primer for the 5' half of the linker as well as the Ricin1729PstI primer (Ricin1729-PstI: 5-GTAGGCGCTGCAGATAACTTGCTGTCCTTCAG-3 (SEQ ID No. 142)) and a mutagenic primer for the 3' half of the linker. The cycling conditions used for the PCRs were 98 degrees C for 2 min.; 98C 1 min., 52C 1 min., 72C 1 min. 15 sec. (30 cycles); 72 degrees C 10min.; 4 degrees C soak. The PCR products were then digested by EcoRI and PstI respectively, electrophoresed on an agarose gel, and the bands purified by via glass wool spin columns. Triple ligations comprising the PCR product pairs (corresponding halves of the new linker) and pVL1393 vector digested with EcoRI and PstI were carried out. Recombinant clones were identified by restriction digests of plasmid miniprep DNA and the altered linkers confirmed by DNA sequencing. See Figure 45 as an example of the cloning strategy. Recombinant clones were identified by restriction digests of plasmid miniprep DNA and the altered linkers confirmed by DNA sequencing. Note that since all altered linker variants were cloned directly into the pVL1393 vector odd-numbered pAPs were no longer required or produced.

Isolation of Recombinant Baculoviruses

Insect cells *S. frugiperda* (Sf9), and *Trichoplusia ni* (Tn368 and BTI-TN-581-4 (High Five)) were maintained on EX-CELL 405 medium (JRH Biosciences) supplemented with 10% total calf serum (Summers et al., A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987)). Two micrograms of recombinant pVL1393 DNA was cotransfected with 0.5 microgram of BaculoGold AcNPV DNA (Pharmingen) into 2 x 10⁶ Tn368 insect cells following the manufacturer's protocol (Gruenwald et al., Baculovirus Expression Vector System: Procedures and Methods Manual, 2nd Edition, (San

Diego, CA, 1993)). On day 5 post-transfection, media were centrifuged and the supernatants tested in limiting dilution assays with Tn368 cells (Summers et al., A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 5 1987)). Recombinant viruses in the supernatants were then amplified by infecting Tn368 cells at a multiplicity of infection (moi) of 0.1, followed by collection of day 3 to 5 supernatants. A total of three rounds of amplification were performed for each recombinant following established procedures (Summers et al., A Manual of Methods of 10 Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987 and Gruenwald et al., Baculovirus Expression Vector System: Procedures and Methods Manual, 2nd Edition, (San Diego, CA, 1993)).

Expression of Mutant Prorcin

15 Recombinant baculoviruses were used to infect 1X10⁷ Tn368 or sf9 cells at an moi of 9 in EX-CELL 405 media (JRH Biosciences) with 25mM α-lactose in spinner flasks. Media supernatants containing mutant proricins were collected 3 or 4 days post-infection.

EXAMPLE 2

20 Harvesting and affinity column purification of pro-ricin variants

Protein samples were harvested three days post transfection. The cells were removed by centrifuging the media at 8288 g for ten minutes using a GS3 (Sorvall) centrifuge rotor. The supernatant was further clarified by centrifuging at 25400 g using a SLA-25 1500 rotor (Sorvall) for 45 minutes. Protease inhibitor phenylmethylsulfonyl fluoride (Sigma) was slowly added to a final concentration of 1mM. The samples were further prepared by adding lactose to a concentration of 20 mM (not including the previous lactose contained in the expression medium). The samples were concentrated 30 to 700 mL using a Prep/Scale-TFF Cartridge (2.5ft, 10K regenerated cellulose (Millipore)) and a Masterflex pump. The samples were then

- 68 -

dialysed for 2 days in 1X Column Buffer (50 mM Tris, 100 mM NaCl, 0.02% NaN₃, pH 7.5) using dialysis tubing (10 K MWCO, 32 mm flat width(Spectra/Por)). Subsequently, the samples were clarified by centrifuging at 25400 g using a SLA-1500 rotor (Sorvall) for 45 minutes.

5 Following centrifugation, the samples were degassed and applied at 4 degrees C to a XK26/20 (Pharmacia) column (attached to a Pharmacia peristaltic pump, Pharmacia Single-path Monitor UV-1 Control and Optical Units, and Bromma LKB 2210 2-Channel Recorder) containing 20 mL of α -Lactose Agarose Resin (Sigma). The column was
10 washed for 3 hours with 1X Column buffer. Elution of pro-ricin variant was performed by eluting with buffer (1X Column buffer (0.1% NaN₃), 100 mM Lactose) until the baseline was again restored. The samples were concentrated using an Amicon 8050 concentrator (Amicon) with a YM10 76 mm membrane, utilizing argon gas to pressurize the chamber.
15 The samples were further concentrated in Centricon 10 (Millipore) concentrators according to manufacturer's specifications.

Purification of Variant pAP-Protein by gel filtration chromatography

In order to purify the pro-ricin variant from processed material produced during fermentation, the protein was applied to a
20 SUPERDEX 75 (16/60) column and SUPERDEX 200 (16/60) column (Pharmacia) connected in series equilibrated with 50 mM Tris, 100mM NaCl, pH 7.5 containing 100 mM Lactose and 0.1% β -mercaptoethanol (β ME). The flow rate of the column was 0.15 mL/min and fractions were collected every 25 minutes. The UV (280 nm) trace was used to
25 determine the approximate location of the purified pAP-protein and thus determine the samples for Western analysis.

Western analysis of column fractions

Fractions eluted from the SUPERDEX columns (Pharmacia) were analyzed for purity using standard Western blotting
30 techniques. An aliquot of 10 μ L from each fraction was boiled in 1X sample buffer (62.6 mM Tris-C1, pH 6.8, 4.4% β ME, 2% sodium dodecyl

- 69 -

sulfate (SDS), 5% glycerol (all from Sigma) and 0.002% bromophenol blue (Biorad)) for five minutes. Denatured samples were loaded on 12% Tris-Glycine Gels (Biorad) along with 50 ng of RCA₆₀ (Sigma) and 5 µL of kaleidoscope prestained standards (Biorad). Electrophoresis was
5 carried out for ninety minutes at 100V in 25 mM Tris-Cl, pH 8.3, 0.1% SDS, and 192 mM glycine using the BioRad Mini Protean II cells (Biorad).

Following electrophoresis gels were equilibrated in transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS, and 20%
10 Methanol) for a few minutes. PVDF Biorad membrane was presoaked for one minute in 100% methanol, rinsed in ddH₂O and two minutes in transfer buffer. Whatman paper was soaked briefly in transfer buffer. Five pieces of Whatman paper, membrane, gel, and another five pieces of Whatman paper were arranged on the bottom cathode (anode) of the
15 Pharmacia Novablot transfer apparatus (Pharmacia). Transfer was for one hour at constant current (2 mA/cm²).

Transfer was confirmed by checking for the appearance of the prestained standards on the membrane. Non-specific sites on the membrane were blocked by incubating the blot for thirty minutes in 1X
20 Phosphate Buffered Saline (1X PBS; 137 mM NaCl, 2.7 mM KC1, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) with 5% skim milk powder (Carnation). Primary antibody (Rabbit α-ricin, Sigma) was diluted 1:3000 in 1X PBS containing 0.1% Tween 20 (Sigma) and 2.5% skim milk and incubated with blot for forty five minutes on a orbital shaker (VWR).
25 Non-specifically bound primary antibody was removed by washing the blot for ten minutes with 1X PBS containing 0.2% Tween 20. This was repeated four times. Secondary antibody donkey anti-rabbit (Amersham) was incubated with the blot under the same conditions as the primary antibody. Excess secondary antibody was washed as
30 described above. Blots were developed with the ECL Western Blotting detection reagents according to the manufacturer's instructions. Blots

- 70 -

were exposed to Medtec's Full Speed Blue Film (Medtee) or Amersham's ECL Hyperfilm (Amersham) for one second to five minutes. Film was developed in a KODAK Automatic Developer.

Determination of lectin binding ability of pro-ricin variant

5 An Immulon 2 plate (VDVR) was coated with 100 µl per well of 10µg/ml of asialofetuin and left overnight at 4°C. The plate was washed with 3X 300 µL per well with ddH₂O using an automated plate washer (BioRad). The plate was blocked for one hour at 37°C by adding 300 µL per well of PBS containing 1% ovalbumin. The plate was
10 washed again as above. Pro-ricin variant pAP-protein was added to the plate in various dilutions in 1X Baculo. A standard curve of RCA₆₀ (Sigma) from 1-10 ng was also included. The plate was incubated for 1 h at 37°C. The plate was washed as above. Anti-ricin monoclonal antibody (Sigma) was diluted 1:3000 in 1X PBS containing 0.5%
15 ovalbumin and 0.1% tween-20, added at 100 µL per well and incubated for 1 h at 37°C. The plate was washed as above. Donkey-anti rabbit polyclonal antibody was diluted 1:3000 in 1X PBS containing 0.5% ovalbumin, 0.1% Tween-20, and added at 100µL per well and incubated for 1 h at 37°C. The plate was given a final wash as described above.
20 Substrate was added to plate at 100µL per well (1 mg/ml o-phenylenediamine (Sigma), 1 µL/ml H₂O₂, 25 µL of stop solution (20% H₂SO₄) was added and the absorbance read (A490nm-A630nm) using a SPECTRA MAX 340 plate reader (Molecular Devices).

Determination of pAP -Protein activity using the rabbit reticulocyte assay

Ricin samples were prepared for reduction.

A) $RCA_{60} = 3,500 \text{ ng}/\mu\text{L of } RCA_{60} + 997 \mu\text{L 1xEndo buffer}$
 $(25\text{mM Tris}, 25\text{mM KCl}, 5\text{mM MGCl}_2, \text{pH 7.6})$

Reduction = 95 µL of 10ng/µL + 5 µL β-mercaptoethanol

B) Ricin variants

Reduction = 40 µL variant + 2 µL β-mercaptoethanol

The ricin standard and the variants were incubated for 30 minutes at room temperature.

5 Ricin - Rabbit Reticulocyte lysate reaction

The required number of 0.5 mL tubes were labelled. (2 tubes for each sample, + and - aniline). To each of the sample tubes 20 µL of 1X endo buffer was added, and 30 µL of buffer was added to the controls. To the sample tubes either 10 µL of 10ng/µL Ricin or 10µL of variant was added. Finally, 30µL of rabbit reticulocyte lysate was added to all the tubes. The samples were incubated for 30 minutes at 30°C using the thermal block. Samples were removed from the eppendorf tube and contents added into a 1.5 mL tube containing 1 mL of TRIZOL (Gibco). Samples were incubated for 15 minutes at room temperature.

15 After the incubation, 200 µL of chloroform was added, and the sample was vortexed and spun at 12,000 g for 15 minutes at 4°C. The top aqueous layer from the samples was removed and contents added to a 1 mL tube containing 500 µL of isopropanol. Samples were incubated for 15 minutes at room temperature and then centrifuged at 12,000 for 15

20 minutes at 4°C. Supernatant was removed and the pellets were washed with 1 mL of 70% ethanol. Centrifugation at 12,000 g for 5 minutes at 4°C precipitated the RNA. All but approximately 20 µL of the supernatant was removed and air dried. Pellets from the other samples (+aniline samples) were dissolved in 20 µL of DEPC treated ddH₂O. An

25 80 µL aliquot of 1 M aniline (distilled) with 2.8 M acetic acid was added to these RNA samples and transferred to a fresh 0.5 mL tube. The samples were incubated in the dark for 3 minutes at 60°C. RNA was precipitated by adding 100 µL of 95% ethanol and 5µL of 3M sodium acetate, pH 5.2 to each tube and centrifuging at 12,000 g for 30 minutes at

- 72 -

4°C. Pellets were washed with 1 mL 70% ethanol and centrifuged again at 12,000g for 5 minutes at 4°C to precipitate RNA. The supernatant was removed and air dried. These pellets were dissolved in 10µL of 0.1 X E buffer. To all samples, 10 µL of formamide loading dye was added. The 5 RNA ladder (8 µL of ladder + 8 µL of loading dye) was also included. Samples were incubated for 2 minutes at 70°C on the thermal block. Electrophoresis was carried out on the samples using 1.2% agarose, 50% formamide gels in 0.1X E buffer + 0.2% SDS. The gel was run for 90 minutes at 75 watts. RNA was visualized by staining the gel in 1 µg/µL 10 ethidium bromide in running buffer for 45 minutes. The gel was examined on a 302 nm UV box, photographed using the gel documentation system and saved to a computer disk.

Results:

Protein Expression Yields

15 Aliquots were taken at each stop of the harvesting/purification and tested. Yields of functional ricin variant were determined by ELISA. Typical results of an 2400 mL prep of infected *T. ni* cells are given below.

	<u>Aliquot</u>	<u>µg pAP 220</u>
20	Before concentration and dialysis	6000
	After concentration and dialysis	4931
	alpha- Lactose agarose column flow through	219
	alpha- Lactose agarose column elution	1058
25	Yield: 1058/6000 = 17.6%	

Purification of pAP -Protein and Western Analysis of column fractions

Partially purified pAP-protein was applied to Superdex 75 and 200 (16/60) columns connected in series in order to remove the

- 73 -

contaminating non-specifically processed pAP-protein. Eluted fractions were tested via Western analysis as described above and the fractions containing the most pure protein were pooled, concentrated and re-applied to the column. The variant was applied a total of three times to 5 the column. Final purified pAP-protein has less than 1% processed variant.

The purified pAP-protein was tested for susceptibility to cleavage by the particular protease and for activation of the A-chain of the proricin variant, (inhibition of protein synthesis). Typically, pAP-protein 10 was incubated with and without protease for a specified time period and then electrophoresed and blotted. Cleaved pAP will run as two 30 kDa proteins (B is slightly larger) under reducing (SDS-PAGE) conditions. Unprocessed pAP-protein, which contains the linker region, will run at 60 kDa.

15 **Activation of pAP -Protein variant with Specific Protease**

Activation of protease treated pAP-protein is based on the method of *May et al.* (EMBO Journal. 8 301-8, 1989). Activation of ricin A chain upon cleavage of the intermediary linker results in catalytic depurination of the adenosine 4325 residue of 28S or 26S rRNA. This 20 depurination renders the molecule susceptible to amine-catalyzed hydrolysis by aniline of the phosphodiester bond on either side of the modification site. The result is a diagnostic 390 base band. As such, reticulocyte ribosomes incubated with biochemically purified ricin A chain, released the characteristic RNA fragment upon aniline treatment 25 of isolated rRNA (May, M.J. et al. Embo. Journal, 8:301-308 at 302-303 (1989)). It is on this basis that the assay allows for the determination of activity of a ricin A chain which has been cleaved from the intact unit containing a particular variant linker sequence.

EXAMPLE 3

30 **In Vitro Protease Digestion of Proricin Variants:**

Affinity-purified proricin variant is treated with individual disease-specific proteases to confirm specific cleavage in the linker

- 74 -

region. Ricin-like toxin variants are eluted from the lactose-agarose matrix in protease digestion buffer (50mM NaCl, 50mM Na-acetate, pH 5.5, 1mM dithiothreitol) containing 100mM lactose. Prorcin substrate is then incubated at 37°C for 60 minutes with a disease-specific protease.

5 The cleavage products consisting ricin A and B chains are identified using SDS/PAGE (Sambrook et al., Molecular Cloning: a Laboratory Manual, 2nd. ed., Cold Spring Harbor Press, 1989), followed by Western blot analysis using anti-ricin antibodies (Sigma).

Cathepsin B may be obtained from Medcor or Calbiochem.

10 Matrix metalloproteinases may be prepared substantially as described by Lark, M.W. et al. (*Proceedings of the 4th International Conference of the Imflammation Research Association Abstract* 145 (1988)) and Welch, A.R. et al. (*Arch. Biochem. Biophys.* 324:59-64 (1995)). Candida acid protease may be prepared substantially as described in Remold, H.H. et
15 al. (*Biochim. Biophys. Acta* 167:399-406 (1968)), Ray, T.L. and Payne, C.D. (*Infect. Immunol.* 58:508-514 (1990)) and Fusek, M. et al. (*FEBS Lett.* 327:108-112 (1993)). Hepatitis A protease may be prepared as described in Jewell, D.A. et al. (*Biochemistry* 31:7862-7869 (1992)). Plasmodium proteases may be prepared as described in Goldberg, D.E. et al. (*J. Exp.
20 Med.* 173:961-969 (1991)) and Cooper, J.A. and Bujard, H. (*Mol. Biochem. Parasitol.* 56:151-160 (1992)).

In Vitro Cytotoxicity Assay:

Human ovarian cancer cells (e.g. MA148) are seeded in 96-well flat-bottom plates and are exposed to ricin-like toxin variants or control
25 medium at 37°C for 16 h. The viability of the cancer cells is determined by measuring [³⁵S]methionine incorporation and is significantly lower in wells treated with the toxin variants than those with control medium.

In Vivo Tumour Growth Inhibition Assay:

30 Human breast cancer (e.g. MCF-7) cells are maintained in suitable medium containing 10% fetal calf serum. The cells are grown, harvested and subsequently injected subcutaneously into

ovariectomized athymic nude mice. Tumour size is determined at intervals by measuring two right-angle measurements using calipers. In animals that received ricin-like toxin variants containing the matrix metalloproteinase-sensitive linkers, tumour size and the rate of 5 tumour growth are lower than animals in the control group.

In Vivo Tumour Metastasis Assay:

The metastasis study is performed substantially as described in Honn, K.V. et al. (*Biochem. Pharmacol.* 34:235-241 (1985)). Viable B16a melanoma tumour cells are prepared and injected subcutaneously into 10 the left axillary region of syngeneic mice. The extent of tumour metastasis is measured after 4 weeks. The lungs are removed from the animals and are fixed in Bouin's solution and macroscopic pulmonary metastases are counted using a dissecting microscope. In general without therapeutic intervention, injection of 10^5 viable tumour cells 15 forms approximately 40-50 pulmonary metastases. The number of metastases in animal treated with proricin variants containing cathepsin B-sensitive linkers is substantially lower.

EXAMPLE 4

In Vitro Protease Digestion of Proricin Variants by Cancer Proteases

20 Cathepsin B or MMP-9

The general protocol for proricin digestion by cancer proteases is described in Examples 2 and 3.

In Vitro Protease Digestion of Cathepsin B Proricin Variant

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region. The proricin substrate is digested in a Cathepsin B protease buffer (50 mM Sodium acetate, 2 mM EDTA, 0.05% Triton) at 40°C. Two hours and overnight (16 hr) digestion reactions are carried out using 100ng of proricin substrate and 100 and 618 ng of Cathepsin B protease per reaction (CALBIOCHEM, USA). The cleavage products of proricin (ricin A and B chains) are identified using SDS/PAGE (Sambrook et al., Molecular cloning: a laboratory Manual, 2nd. ed., Cold Spring Harbor

Press, 1989), followed by Western blot analysis using anti-ricin antibodies (Sigma).

In Vitro Protease Digestion of MMP-9 Proricin Variant

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region. The proricin substrate is digested in 1X column buffer (100 mM NaCl, 50 mM Tris, PH 7.5) at 37°C. Two hours and overnight (16 hr) digestion reactions are set up using 50 ng of MMP-9 proricin substrate and 20 and 200 ng of MMP-9 protease per reaction (CALBIOCHEM, USA). The cleavage products of proricin (ricin A and B chains) are identified using SDS/PAGE (Sambrook et al., Molecular cloning: a laboratory Manual, 2nd. ed., Cold Spring Harbor Press, 1989), followed by Western blot analysis using anti-ricin antibodies (Sigma).

The protocol for Western analysis of ricin chains is described in Example 2.

Results

Figures 48 and 49 illustrate Western blots showing the cleavage of the protease-sensitive linkers by cathepsin B (pAP 214) and MMP-9 (pAP 220) respectively. Without protease digestion, the proricin variant appears as a single band at approximately 60 kDa (Lane B of Figure 48 and Lane A of Figure 49). Wild type ricin A chain and B chain appear as two disparate bands at approximately 30 kDa (Lane A of Figure 48 and Lane E of Figure 49). Increasing extent of proricin cleavage can clearly be observed with increasing protease concentration (Lanes C and D of Figure 48 and Lanes B-C of Figure 49).

EXAMPLE 5

In vitro protease digestion of various proricin variants by their corresponding proteases.

The general protocol for proricin digestion by coresponding proteases was as desribed in Examples 2 and 3 and should be considered in connection with the digestions described below.

Cleavage of pAP-222 protein with the Matrix Metalloproteinase 2 (MMP-2)

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker
5 region.

The pAP-222 protein sample (1.0 ug) was digested with the MMP-2 protease (1.0 ug) overnight at 37° C. The total volume of the digestion reaction was 21.5 ul, and 0.250 ug of the reaction sample was loaded on a protein gel. The MMP-2 protease was purchased from
10 Calbiochem-Novabiochem Corporation, USA.

Cleavage of pAP-248 protein with the Human Cytomegalovirus (HCMV) protease

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker
15 region.

The pAP-248 protein sample (1.19 ug) was digested with the HCMV protease (1.13 ug) overnight at 37°C. The total volume of the digestion was 10.5 ul, and 0.279 ug of the reaction sample was loaded on a protein gel. The HCMV was purchased from BACHEM Bioscience Inc., USA.

20 **Cleavage of pAP-256 protein with the Hepatitis A virus 3C (HAV 3C) protease**

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region.

25 The pAP-256 protein sample (1.26 ug) was digested with the HAV 3C protease (5 ug) overnight at 37°C. The total volume of the digestion was 12.5 ul, and 0.302 ug of the digestion sample was loaded on a protein gel. The HAV 3C protease was a gift from Dr. G. Lawson from Bates Collage, Main, USA.

30 **Cleavage of pAP-270 protein with the Matrix Metalloproteinase 2 (MMP-2)**

- 78 -

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region.

The pAP-270 protein sample (0.120 ug) was digested with the MMP-2

5 protease (0.25 ug) overnight at 37° C. The total volume of the digestion reaction was 22.5 ul, and 0.106 ug of the reaction sample was loaded on a protein gel. The MMP-2 protease was purchased from Calbiochem-Novabiochem Corporation, USA.

Cleavage of pAP-288 protein with tPA plasminogen tissue activator

10 Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region. The pAP-288 protein sample (1.65 ug) was digested with the t-PA protease (0.5 ug) overnight at 37° C. The total volume of the digestion reaction was 55 ul, and 0.6 ug of the reaction sample was
15 loaded on a protein gel. The t-PA was purchased from Sigma Chemical Co., USA.

Cleavage of pAP-294 protein with human neutrophil elastase

20 Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region.

The pAP-256 protein sample (0.6 ug) was digested with the Elastase protease (5 ug) at 25° C for one hour. The total volume of the digestion reaction was 52.5 ul, and 0.171 ug of the digestion sample was loaded on a protein gel. The Human Neutrophil Elastase protease was purchased
25 from Cedarlane Laboratories Limited, Canada.

Cleavage of pAP-296 protein with calpain

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region. The pAP-296 protein sample (2.05 ug) was digested with the
30 Calpain protease (10 ug) overnight at 37° C. The total volume of the digestion reaction was 35 ul and 0.761 ug of the reaction sample was

- 79 -

loaded on a protein gel. The Calpain protease was purchased from Sigma Chemical Co., USA

Results

Figures 52, 54, 58 & 66(MMP-2), 60, 64 and 62 show the cleavage 5 of proteases of linkers by HCMV, HAV 3C, MMP-2, t-PA, calpain, and human neutrophil elastase respectively. Without protease digestion, the proricin variants appear as a single band at approximately 60kDA (Lane A in connection with Figure 52; Lane B of Figure 54; Lane A of Figure 58; Lane B of Figure 60; and Lane C of Figure 62; lane B of Figure 10 64 and lane B of Figure 66). Wild type ricin chain A and B appear as two bands at approximately 30kDA (see for example Lanes C and D of Figure 52) proricin cleavage can clearly be observed with the appearance of 30kDA bands in connection with the protein which has been digested by the respective protease (see Lane B of Figure 52; Lane C of Figure 54; or 15 Lane B of Figure 58 for examples).

EXAMPLE 6

In Vitro Translation Assay (Activation by Cancer Proteases Cathepsin B or MMP-9)

The general protocol for the rabbit reticulocyte lysate reaction to 20 test the cytotoxicity of cancer protease-activated proricin is described briefly in Example 3 and is described in more detail in Example 2.

Results

Activation of pAP 214 and pAP 220 proricin variants by cathepsin B and MMP-9, based on the method of May et al. (EMBO J. 25 8:301-308, 1989), is illustrated in Figures 50 and 51 respectively. The appearance of the 390 base pair product (positive control) is observed in Lane F of Figure 50 and Lane G of Figure 51. This 390 base pair product is absent in the negative control lanes. Without cathepsin or MMP-9 activation, no or minimal N-glycosidase activity in the pAP 214 variant 30 (Lanes H to L, Figure 50) or the pAP 220 variant (Lanes A to E, Figure 51) was observed. When the pAP 214 variant and the pAP 220 variant were activated by cathepsin or MMP-9 respectively, appearance of the 390 base

- 80 -

pair product was observed in a proricin concentration-dependent manner (Lanes A to E of Figure 50 and Lanes H to L of Figure 51). The present experimental series demonstrated the successful and selective activation of proricin variants by cancer-associated proteases.

5 **EXAMPLE 7**

The general protocol for the rabbit retoculocyte lysate reaction is described briefly in Example 3 and is described in more detail in Example 2, all of which compliments the description below.

10 **Depurination of Rabbit Reticulocyte 28S Ribosomal RNA by Digested and Undigested Ricin Variants**

Affinity-purified mutant proricin mutants which were previously digested with the disease-specific protease, were reduced with 5% 2-mercaptoethanol then diluted to 100ng, 14.2ng, 2.0ng, 291pg, and 41.7pg with 1 X ENDO buffer(25mM Tris pH 7.6, 25mM KCl, 5mM 15 MgCl₂) and incubated with rabbit reticulocyte lysate, untreated (Promega) for 30minutes at 30°C. To compare the digested with the undigested proricin variant, the proricin in digestion buffer (according to the specific digestion protocol) was treated in the same manner as the digested sample. As a positive and negative control, 10ng of ricin A 20 chain and 1 X ENDO buffer consecutively, was incubated with rabbit reticulocyte lysate, untreated, for 30 min at 30°C.

Aniline Cleavage of rRNA and Gel Fractionation

Total RNA was then extracted from reticulocyte lysate translation mixtures with Trizol reagent (Gibco-BRL) as per 25 manufacturer's instructions. The RNA was incubated with 80ul of 1M aniline (distilled) with 2.8M acetic acid for 3 min at 60°C in the dark. Ethanol-precipitated RNA samples were dissolved in 20ul of 50% formamide, 0.1X E buffer (3.6mM Tris, 3mM NaH₂PO₄, 0.2mM EDTA), and 0.05% xylene cyanol. 10ul of this was heated to 70°C for 2 minutes, 30 loaded and electrophoresed in 1.2% agarose, 0.1X E buffer, and 50% formamide gel with RNA running buffer (0.1 X E buffer, 0.2% SDS).

Results

Activation of pAP-248 proricin variant by HCMV; pAP-256 by HAV3C protease; pAP-270 by MMP-2 protease; pAP-288 by t-PA protease; pAP-294 by human neutrophil elastase; pAP-296 by calpain; and pAP-222 by MMP-2 is illustrated in Figures 52, 55, 59, 61, 63, 65, and 67 respectively. The appearance of the 390 base pair product (deposit of control) is observed in lane L of Figures 53, 55, 61, 63, 65 and 67. The 390 base pair product is observed in lane A of Figures 59 (activation of pAP-270 by MMP-2). This 390 base pair product is absent in the negative control lanes. Without the specific protease activation, no or minimal activity is seen in the lanes which contained only the proricin variant without digestion (see lane A, B, C, D, and E of Figures 53, 55, 61, 63, 65, and 67). The same observation is made in connection with pAP-270 in Figure 59, however, the undigested lanes appear as H, I, J, K and L. When the variant was activated by its respective protease, there is an appearance of the 390 base pair product in a proricin concentration-dependent manner (see Lanes H, I, J, K and L of Figure 53, 55, 61, 63, 65, and 67 and Lanes A, B, C, D, and E of Figure 59). The present experimental series demonstrate the successful and selective activation of the identified proricin variants by selective corresponding proteases.

20 EXAMPLE 8

Procedure for Examining the Cytotoxicity of Ricin and Ricin Variants on the COS-1 Cell Line

Cell Preparation

After washing with 1XPBS (0.137 M NaCl, 2.68 mM KCl, 8.10 mM Na₂HPO₄, 1.47 mM KH₂PO₄), cells in log phase growth were removed from plates with 1X trypsin/EDTA (Gibco/BRL). The cells were centrifuged at 1100 rpm for 3 min, resuspended in Dulbecco's Modified Eagle Medium containing 10%FBS and 1X pen/strep, and then counted using a haemocytometer. They were adjusted to a concentration of 5 X 10⁴ cells•ml⁻¹. One hundred microliters per well of cells was added to wells 2B - 2G through to wells 9B - 9G of a Falcon 96 well tissue culture plate. A separate 96 well tissue culture plate was

- 82 -

used for each sample of Ricin or Ricin variant. The plates were incubated at 37(C with 5% CO₂ for 24 hours.

Toxin Preparation

The Ricin and Ricin variants were sterile filtered using a 0.22μm filter (Millipore). The concentration of the sterile samples were then quantified by A₂₈₀ and confirmed by BCA measurements (Pierce). For the variants digested with the protease in vitro, the digests were carried out as described in the digestion procedure for each protease. The digests were then diluted in the 1000 ng•ml⁻¹ dilution and sterile filtered. The Ricin and the undigested pAP214 in the pAP 214 cytotoxicity data were treated in the same manner but without the Cathepsin B treatment. Ricin and Ricin variants were serially diluted to the following concentrations: 1000 ng•ml⁻¹, 100 ng•ml⁻¹, 10 ng•ml⁻¹, 1 ng•ml⁻¹, 0.1 ng•ml⁻¹, 0.01 ng•ml⁻¹, 0.001 ng•ml⁻¹ with media containing 10%FBS and 1X pen/strep.

Application of Toxin or Variants to Plates

Columns 2 to 9 were labeled: control, 1000 ng•ml⁻¹, 100 ng•ml⁻¹, 10 ng•ml⁻¹, 1 ng•ml⁻¹, 0.1 ng•ml⁻¹, 0.01 ng•ml⁻¹, 0.001 ng•ml⁻¹ consecutively. The media was removed from all the sample wells with a multichannel pipettor. For each plate of variant and toxin, 50μl of media was added to wells 2B to 2G as the control, and 50μl of each sample dilution was added to the corresponding columns. For the pAP220 + MMP-9 data, the plates were incubated for one hour at 37(C with 5% CO₂, then washed once and replaced with media, then incubated for 48 hours at 37(C with 5% CO₂. For the pAP 214 + Cathepsin B data, the toxin was left on the plates and incubated for 24 hours at 37(C with 5% CO₂, then 50 μl of media was added to the wells with the toxin and incubated for another 24 hours at 37(C with 5% CO₂.

Sample Application

The whole amount of media (and/or toxin)was removed from each well with a multichannel pipettor, and replaced with 100 µl of the substrate mixture (Promega Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit). The plates were incubated at 37(C with 5% CO₂

5 for 2 to 4 hours, and subsequently read with a Spectramax 340 96 well plate reader at 490nm. The IC₅₀ values were calculated using the GRAFIT software program.

Results

In experiments with pAP-214 and Cathepsin B incubated with

10 COS-1 cells, it may be seen that cells incubated with pAP-214 alone, pAP-214 was ineffective at causing cell death (see Figure 56). However, the cytotoxicity of pAP-214 digested with Cathepsin B behaves similarly to the ricin control in COS-1 cells. This is also illustrated in Figure 56. Similarly, the cytotoxicity of undigested pAP-220 when incubated with

15 COS-1 cells is lower than the cytotoxicity observed with COS-1 cells incubated with pAP-220 digested with MMP-9. Indeed the results suggest that the toxicity of digested pAP-220 is greater than that of ricin. (See Figure 57).

EXAMPLE 9

20 Procedure for Examining the Cytotoxicity of Ricin and Ricin Variants on Various Tissue Culture Cell Lines

Cell Preparation

After washing with 1XPBS (1.37M NaCl, 26.8mM KCl, 81mM Na₂HPO₄, 14.7mM KH₂PO₄), cells in log phase growth were removed

25 from plates with 1X trypsin/EDTA (Gibco/BRL). The cells were centrifuged at 1100 rpm for 3 min, resuspended in media containing 10%FBS and 1X pen/strep (media used depended on the cell line being tested), and then counted using a haemocytometer. They were adjusted to a concentration of 5 X 10⁴ cells•ml⁻¹ (faster growing cell lines were

30 adjusted to 2 X10⁴ cells•ml⁻¹). One hundred microliters per well of cells was added to wells 2B - 2G through to wells 9B - 9G of a Falcon 96 well

- 84 -

tissue culture plate. A separate 96 well tissue culture plate was used for each sample of Ricin or Ricin variant. The plates were incubated at 37(C with 5% CO₂ for 24 hours.

Toxin Preparation

5 The Ricin and Ricin variants were sterile filtered using a 0.22µm filter (Millipore). The concentration of the sterile samples were then quantified by A₂₈₀ and confirmed by a BCA measurement (Pierce). Ricin and Ricin variants were serially diluted to the following concentrations: 3000 ng•ml⁻¹, 300 ng•ml⁻¹, 30 ng•ml⁻¹, 3 ng•ml⁻¹, 0.3 ng•ml⁻¹,
10 0.03ng•ml⁻¹, 0.003 ng•ml⁻¹ with media containing 10%FBS and 1X pen/strep.

Application of Toxin or Variants to Plates

Columns 2 to 9 were labeled: control, 0.001 ng•ml⁻¹, 0.01 ng•ml⁻¹, 0.1 ng•ml⁻¹, 1ng•ml⁻¹, 10 ng•ml⁻¹, 100 ng•ml⁻¹, 1000 ng•ml⁻¹ consecutively. For each plate of variant and toxin, 50µl of media was added to wells 2B to 2G as the control, and 50µl of each sample dilution was added to the corresponding columns containing 100µl per well of cells (i.e. 50 µl of the 3000 ng•ml⁻¹ dilution added to the wells B-G in column 9, labeled 1000 ng•ml⁻¹). The plates were incubated for 48 hours at 37(C with 5% CO₂.

Sample Application

An amount of 140µl was removed from each well with a multichannel pipettor, and replaced with 100 µl of the substrate mixture (Promega Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit). The plates were incubated at 37(C with 5% CO₂ for 2 to 4 hours, and subsequently read with a Spectramax 340 96 well plate reader at 490nm. The IC₅₀ values were calculated using the GRAFIT software program.

Results

- 85 -

Referring to Table 2, it may be seen that the survival of cells is correlated with the proricin variant and the cell specific protease produced by the cell type. For example, in the HT1080 cell line, both pAP-214 and pAP-220 required only 2-1/2 times the amount of ricin to achieve the same level of cytotoxicity. On the other hand, pAP-224 required 193 times the amount of ricin to achieve the same level of cell death. As well, it may be seen that in the cells where expression of Cathepsin D is found, pAP-214 and 220 were more effective at causing cell death than ricin and more effective than pAP-224. Details concerning the various cells types used in these experiments are outlined below.

COS-1 (African Green Monkey Kidney Cells)

This is an SV40 transformed cell line which was prepared from established simian cells CV-1. (Reference: Gluzman, Y. (1975) Cell, 23, 175 - 182)(ATCC CRL 1650)

HT-1080 Human Fibrosarcoma

(ATCC CCL 121) This cell line was shown to produce active MMP-9 in tissue culture. References: Moore et al. (1997) Gynecologic Oncology 65, 83-88.

9L Rat Glioblastoma

Glioblastomas are generally associated with cathepsin B expression. Levels of cathepsin B expression correspond to the extent of progression of malignancy i.e. highest levels for glioblastomas over anaplastic astrocytomas over low-grade gliomas and normal brain tissue. The 9L cell line was provided by Dr. William Jia of the B.C. Cancer Agency.

References: Mikkelsen et al. (Aug. 1995) Journal of Neurosurgery 83(2), 285-290. Nakano et al. (1995) J. of Neurosurgery 83(2), 298-307.

MCF-7 Human Breast Cancer Cell Line (Epithelial)

(ATCC CRL 1555) In the absence of estrogen cathepsin B has not been shown to be elevated relative to normal cells. It can be induced with estrogen to produce Cathepsin D. Production of MMP-9 is unknown.

- 86 -

Having illustrated and described the principles of the invention in a preferred embodiment, it should be appreciated by those skilled in the art that the invention can be modified in arrangement and detail without departure from such principles. We claim all modifications 5 coming within the scope of the following claims.

All publications, patents and patent applications referred to herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in 10 its entirety.

**FULL CITATIONS FOR CERTAIN REFERENCES REFERRED TO IN
THE SPECIFICATION**

Bever Jr., C.T., Panitch, H.S., and Johnson, K.P. (1994) Neurology 44(4), 745-8. Increased cathepsin B activity in peripheral blood mononuclear 5 cells of multiple sclerosis patients.

Cohen, P., Graves, H.C., Peehl, D.M., Kamarei, M., Giudice, L.C., and Rosenfeld, R.G. (1992) Journal of Clinical Endocrinology and Metabolism 75(4), 1046-53. Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma.

10 Conover, C.A. and De Leon, D.D. (1994) J. Biol. Chem. 269(10), 7076-80. Acid activated insulin-like growth factor-binding protein-3 proteolysis in normal and transformed cells. Role of cathepsin D.

Hansen, G., Schuster, A., Zubrod, C., and Wahn, V. (1995) Respiration 62(3), 117-24. Alpha 1-proteinase inhibitor abrogates proteolytic and 15 secretagogue activity of cystic fibrosis sputum.

Muller, H.L., Oh, Y., Gargosky, S.E., Lehrnbecher, T., Hintz, R.L., and Rosenfeld, R.G. (1993) Journal of Clinical Endocrinology and Metabolism 77(5), 1113-9. Concentrations of insulin-like growth factor (IGF)-binding protein-3 (IGFBP-3), IGF, and IGFBP-3 protease activity in 20 cerebrospinal fluid of children with leukemia, central nervous system tumor, or meningitis.

TABLE 1

Table I - Sequence and Location of Oligonucleotide Primers

Name of Primer	Primer Sequence †	Corresponds to preproricin nucleotide numbers: (see Figures 8-10)
Ricin-109	5' - <u>GGAGATGAAACCGGGAGGAAATACTATTGTAAT</u> -3'	27 to 59
Ricin-99Eco	5' - <u>GCGGAATTCCGGGAGGAAATACTATTGTAAT</u> -3'	37 to 59
Ricin267	5' - ACGGTTTATTTAGTTGA -3'	300 to 317
Ricin486	5' - ACTTGCTGGTAATCTGAG -3'	519 to 536
Ricin725	5' - AGAATAGTTGGGGAGAC -3'	758 to 775
Ricin937	5' - AATGCTGATGTTGTATG -3'	970 to 987
Ricin1151	5' - CGGGAGTCTATGTGATGA -3'	1184 to 1201
Ricin1399	5' - GCAAATAGTGGACAAGTA -3'	1432 to 1449
Ricin 1627	5' - GGATTGGTGTAGATGTG -3'	1660 to 1677
Ricin1729C	5' - ATAACTTGCTGTCCTTCA -3'	1864 to 1846
Ricin1729C Xba	5' - <u>CGCTCTAGATAACTTGCTGTCCTTCA</u>	1864 to 1846

† Underlined sequences inserted for subcloning purposes and not included in final preproricin sequences

Table 2: Comparative Toxicities to Selected Cell Lines of Ricin and Ricin Provariants

<u>Cell Line</u>	<u>IC50_{Ricin}</u> <u>(ng/ml)</u>	<u>IC50_{pAP214}</u> <u>IC50_{Ricin}</u>	<u>IC50_{pAP220}</u> <u>IC50_{Ricin}</u>	<u>IC50_{pAP224}</u> <u>IC50_{Ricin}</u>
COS-1	0.1	17	22	150
HT1080	0.5	2.46	2.14	193
9L	10.8	1.3	1.7	32.3
MCF-7 (without estrogen)	0.09	27.8	40	742

I CLAIM:

1. A purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence linking 5 the A and B chains, the heterologous linker sequence containing a cleavage recognition site for a disease-specific protease.
2. The nucleic acid sequence of claim 1 wherein the linker sequence contains a cleavage recognition site recognized by a protease selected from the group consisting of: a cancer associated protease, a 10 viral protease, a fungal protease, and a parasite protease.
3. A nucleic acid sequence of claim 2 wherein the A chain is ricin A chain, abrin toxin A chain, diphtheria toxin A chain, or Domain I of Pseudomonas endotoxin.
4. A nucleic acid sequence of claim 2 wherein the A chain is 15 volkensin toxin A chain, cholera toxin A chain, modeccin toxin A chain or shiga toxin A chain.
5. A nucleic acid sequence of claim 2 wherein the B chain is ricin B chain, abrin toxin A chain, diphtheria toxin B chain, or Domain II of Pseudomonas endotoxin.
- 20 6. A nucleic acid sequence of claim 2 wherein the B chain is volkensin toxin B chain, cholera toxin B chain, modeccin toxin B chain or shiga toxin B chain.
7. A nucleic acid sequence of claim 2 wherein the cleavage 25 recognition site is recognized by a cancer-associated protease which is selected from the group consisting of: cathepsin B, an Epstein-Barr

- 91 -

virus-specific protease, a matrix metalloproteinase, cathepsin L, cathepsin D, urokinase-type plasminogen activator, tissue-type plasminogen activator, human prostate-specific antigen, kallikrein, neutrophil elastase, and calpain.

5 8. A nucleic acid sequence of claim 2 wherein the cleavage recognition site is recognized by a parasitic protease which is a *Plasmodium falciparum* protease.

9. A nucleic acid sequence of claim 2 wherein the cleavage recognition site is recognized by viral protease which is selected from
10 the group consisting of: human cytomegalovirus, human herpes virus, varicella zoster virus, hepatitis A virus, hepatitis C virus, and infectious laryngotracheitis virus.

10. A nucleic acid sequence of claim 2 wherein the cleavage recognition site is recognized by fungal protease which is a *Candida* acid protease.
15

11. A nucleic acid sequence of claim 2 having the nucleotide sequence according to SEQ ID No. 3; SEQ ID No 5; SEQ ID No 7; SEQ ID No 9; SEQ ID No 11; SEQ ID No 13; SEQ ID No 15; SEQ ID No 17; SEQ ID No 19; SEQ ID No 21; SEQ ID No 23; SEQ ID No 25; SEQ ID No 27;
20 SEQ ID No 29; SEQ ID No 31; SEQ ID No 33; SEQ ID No 35; SEQ ID No 37; SEQ ID No 39; SEQ ID No 48; SEQ ID No 50; SEQ ID No 52; SEQ ID No 54; SEQ ID No 74; SEQ ID No 77; SEQ ID No 80; SEQ ID No 83; SEQ ID No 86; SEQ ID No 89; SEQ ID No 92; SEQ ID No 95; SEQ ID No 98; SEQ ID No 101; SEQ ID No 104; SEQ ID No 107; SEQ ID No 110; SEQ ID No 113; SEQ ID No 116; SEQ ID No 119; SEQ ID No 122; or SEQ ID No 125.

12. A plasmid incorporating the nucleic acid of claim 1 to 11.

13. A baculovirus transfer vector incorporating the nucleic acid of claim 1 to 11.
14. A recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin- like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a disease-specific protease.
5
15. The recombinant protein of claim 14 wherein the linker sequence contains a cleavage recognition site which is recognized by a protease selected from the group consisting of: a cancer, viral, fungal, and a parasitic protease.
10
16. A recombinant protein of claim 14 wherein the A chain is ricin A chain, abrin toxin B chain, diphtheria toxin A chain, or Domain I of Pseudomonas endotoxin.
17. A recombinant protein of claim 14 wherein the A chain is volkensin toxin A chain, cholera toxin A chain, modeccin toxin A chain or shiga toxin A chain.
15
18. A recombinant protein of claim 14 wherein the B chain is ricin B chain, abrin toxin B chain, diphtheria toxin B chain, or Domain II of Pseudomonas endotoxin.
- 20 19. A recombinant protein of claim 14 wherein the B chain is volkensin toxin B chain, cholera toxin B chain, modeccin toxin B chain or shiga toxin B chain.
20. A recombinant protein of claim 14 wherein the cleavage recognition site is recognized by a cancer-associated protease selected

from the group consisting of: cathepsin B, an Epstein-Barr virus-specific protease, a matrix metalloproteinase, cathepsin L, cathepsin D, urokinase-type plasminogen activator, tissue-type plasminogen activator, human prostate-specific antigen, kallikrein, neutrophil
5 elastase, and calpain.

21. A recombinant protein of claim 14 wherein the cleavage recognition site is recognized by a parasitic protease which is a *Plasmodium falciparum* protease.
22. A recombinant protein of claim 14 wherein the cleavage 10 recognition site is recognized by a viral protease which is selected from the group consisting of: human cytomegalovirus, human herpes virus, varicella zoster virus, hepatitis A virus, hepatitis C virus and infectious laryngotracheitis virus.
23. A recombinant protein of claim 14 wherein the cleavage 15 recognition site is recognized by a fungal protease which is a *Candida* acid protease.
24. A recombinant protein of claim 14 having the linker amino acid sequence according to SEQ ID No. 40; SEQ ID No. 41; SEQ ID No. 42; SEQ 20 ID No. 43; SEQ ID No. 44; SEQ ID No. 45; SEQ ID No. 46; SEQ ID No. 55; SEQ ID No. 56; SEQ ID No. 57; SEQ ID No. 58; SEQ ID No. 59; SEQ ID No. 60; SEQ ID No. 61; SEQ ID No. 62; SEQ ID No. 63; SEQ ID No. 64; SEQ ID No. 65; SEQ ID No. 66; SEQ ID No. 67; SEQ ID No. 68; SEQ ID No. 69; SEQ ID No. 70; SEQ ID No. 71; SEQ ID No. 72; SEQ ID No. 75; SEQ ID No. 78; SEQ ID No. 81; SEQ ID No. 84; SEQ ID No. 87; SEQ ID No. 90; SEQ ID 25 No. 93; SEQ ID No. 96; SEQ ID No. 99; SEQ ID No. 102; SEQ ID No. 105; SEQ ID No. 108; SEQ ID No. 111; SEQ ID No. 114; SEQ ID No. 117; SEQ ID No. 120; SEQ ID No. 123; or SEQ ID No. 126.

- 94 -

25. A method of inhibiting or destroying cells affected by a disease, which cells are associated with a protease specific to the disease comprising the steps of:

- (a) preparing a purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin, and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for the protease;
- 5 (b) introducing the nucleic acid into a host cell and expressing the nucleic acid in the host cell to obtain a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a linker amino acid sequence;
- 10 (c) suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient, and
- 15 (d) contacting the cells with the recombinant protein.

26. The method of claim 25 where the disease is one of cancer or cells infected with a fungus, virus or parasite.

27. A method of inhibiting or destroying cells affected by a disease, which cells are associated with a protease specific to the disease comprising the step of contacting the cells with a recombinant protein according to any one of claims 14 to 24.

28. A method of treating a disease comprising administering a recombinant protein according to any one of claims 14 to 24 to an animal in need thereof.

25 29. A method of treating a disease comprising administering a nucleic acid molecule according to any one of claims 2 to 11 to an animal in need thereof.

- 95 -

30. A method of treating a mammal with cancer or infected with a fungus, virus or parasite, comprising the steps of preparing a recombinant protein of claim 14 wherein the linker sequence contains a cleavage recognition site for a cancer, fungal, viral or parasitic protease
5 and administering the protein to the mammal.

31. A process for preparing a pharmaceutical for treating a mammal with cancer, fungal infection, viral infection or parasitic infection, comprising the steps of :

(a) preparing a purified and isolated nucleic acid having a
10 nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin, and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a cancer, viral or parasitic protease;

(b) introducing the nucleic acid into a host cell and expressing
15 the nucleic acid in the host cell to obtain a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a linker amino acid sequence;

(c) suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient.

20 32. A use of a recombinant protein according to any one of claims 14 to 24 to treat a disease.

33 A use of a nucleic acid molecule according to any one of claims 1 to 11 to treat a disease.

25 34. A pharmaceutical composition for treating cancer or a fungal, or viral, or parasitic infection in an animal comprising the recombinant protein of claim 14 and a pharmaceutically acceptable carrier, diluent or excipient.

- 96 -

35. A pharmaceutical composition for treating cancer or a fungal, or viral, or parasitic infection in an animal comprising the nucleic acid molecule of claim 2 and a pharmaceutically acceptable carrier, diluent or excipient.

1/254

FIGURE 1**Complete Sequence of Baculovirus Transfer Vector, pVL1393**

ID PVL1393 preliminary; circular DNA; SYN;
9632 BP.
XX
AC IG1137;
XX
DT 01-FEB-1993 (Rel. 7, Created)
DT 01-JUL-1995 (Rel. 12, Last updated, Version
1)
XX
DE E. coli plasmid vector pVL1393 - complete.
XX
KW cloning vector.
XX
OS Cloning vector
OC Artificial sequences; Cloning vehicles.
XX
RN [1]
RC p2Bac from baculovirus
RC p2Blue from p2Bac
RC pBlueBac from AcNPV
RC pBlueBac2 from AcNPV
RC pBlueBacIII from AcNPV
RC pBlueBacHisA from AcNPV
RC pBlueBacHisB from AcNPV
RC pBlueBacHisC from AcNPV
RC pVL1392, pVL1393 from pAc360
RA ;
RT ;
RL The Digest 5:2-2(1992).
XX
CC NM (pVL1393)
CC CM (yes)
CC NA (ds-DNA)
CC TP (circular)
CC ST ()
CC TY (plasmid)
CC SP (British
Biotechnology) (Invitrogen)
CC HO (E.coli NM522) (E.coli
INValphaF') (insect)
CC CP ()
CC FN (expression) (transfer)
CC SE ()
CC PA (pAC360)
CC BR (pVL1392)
CC OF ()
CC OR ()
XX
FH Key Location/Qualifiers
FH

2/254

FIGURE 1 (Cont'd)

FT misc_feature 0..0
 FT /note="1. pAc360, ori/amp/AcMNPV
 polyhedrin gene
 FT > pVL1393 9632bp"
 FT transposon 0..0
 FT /note="TRN AcMNPV"
 FT misc_binding 868..868
 FT misc_binding /note="SIT SacII"
 1395..1395
 FT misc_binding /note="SIT ApaI"
 1901..1901
 FT misc_binding /note="SIT XhoI"
 0..0
 FT promoter /note="PRO AcMNPV polyhedrin gene"
 FT misc_binding 0..0
 FT /note="MCS
 FT BamHI-SmaI-XbaI-EcoRI-NotI-XmaIII-PstI-
 BglII."
 FT rep_origin 0..0
 FT /note="ORI E. coli pMB1 (ColE1 and
 pBR322)"
 FT CDS complement(0..0)
 FT (bla) /note="ANT E. coli beta-lactamase gene
 XX ampicillin resistance gene (apr/amp)"
 SQ Sequence 9632 BP; 2602 A; 2122 C; 2176 G; 2732 T; 0
 other;

```

aagctttact cgtaaaagcga gttgaaggat catatttagt tgcgtttatg
agataagatt gaaagcacgt gtaaaatgtt tcccgcgcgt tggcacaact
atttacaatg cggccaagtt ataaaaagatt ctaatctgtat atgttttaaa
acacctttgc ggccccgagtt gtttgcgtac gtgactagcg aagaagatgt
gtggaccgcga gaacagatag taaaacaaaa cccttagtatt ggagcaataa
tcgatTTAAC caacacgtct aaatattatg atggtgtgca ttttttgcgg
gcggggcctgt tatacaaaaa aattcaagta cctggccaga ctttgccgcc
tcaaaggcata gttcaagaat ttattgacac ggtaaaaagaa ttacagaaaa
agtgtcccgg catgttggtg ggcgtgcact gcacacacgg tattaatcgc
accggttaca tggtgtgcag atattaatg cacaccctgg gtattgcgcc
gcaggaagcc atagatagat tcgaaaaagc cagaggtcac aaaattgaaa
gacaaaatta cgttcaagat ttattaattt aattaatatt atttgcattc
tttaacaaat actttatcct atttcaaat tgttgcgtt cttccagcga
accaaaaacta tgcttcgctt gctccgtta gctttagcc gatcagtggc
gttgttccaa tcgacggtag gattaggccg gatattctcc accacaatgt
tggcaacggtt gatgttacgt ttatgtttt gttttccac gtacgtctt
tggccggtaa tagccgtaaa cgtagtgcgg tcgcgtca cgcacaacac
cgatgtttg cgcttgcgg cgggtattt aaccgcgcga tccgacaaat
ccaccactt ggcaactaaa tcggtgacct ggcgtctt tttctgcatt
atttcgtctt tctttgcat ggttccctgg aagccggtgt acatgcgggt
tagatcagtc atgacgcgcg tgacctgcaa atcttggcc tcgatctgct
tgtccttcat ggcaacgtat cgttcaataa actctgttt tttaacaagt
tcctcggtt ttgcgcac caccgcttgc agcgcgttt gttgcgtt
aatgtcgca atcagcttag tcaccaactg tttgcgttcc tcctccgggt
gtttgatcgc gggatcgtac ttgcgggtgc agagcactt aggaattact
tcttctaaaa gccattctt gtaattctatg gcgtaaggca atttggactt

```

FIGURE 1 (Cont'd)

cataatcagc tgaatcacgc cggtttagt aatgaggact gatatcggt
 gcaaatacag cgggtcgccc ctttcacga cgctgttaga ggttagggccc
 ccattttgga tggctctgttc aaataacgt ttgttatttat tggctacatg
 aacacgtata gctttatcac aaactgtata tttaaaactg ttagcgacgt
 ccttggccac gaaccggacc tgggtgtcgc gctctagcac gtaccgcagg
 ttgaacgtat cttctccaaa tttaaattct ccaattttaa cgcgagccat
 tttgatacac gttgtgtcgat ttgcacaaa ctatttttt ttaacgc当地
 ctaaacttat tgggttaagc aataattaaa tatggggaa catgc当地
 tacaacactc gtcgttatga acgcacacgg cggccgtctc ggc当地
 gctaaaacgt gttgcgcgtt caacgc当地 aacatcgcaa aagccaatag
 tacagtttg atttgcata taacggcgat tttaaattt atcttattta
 ataaatagtt atgacgccta caactccccg cccgc当地 ctc当地
 ctc当地 ctc当地 gtggccgaaac acgtcgagcg
 ggtggcgat gaccagc当地 gtggccgacg cgc当地 ccatctgtac
 accgaatgat cgtc当地 cgtc当地 aggacacgtcg gc当地
 gcaaattcga aaatatac agttgggtt tttgc当地 tctatcg当地
 cgttggcat gtacgtccga acgttgcattt gcatgc当地 cgc当地
 tcattgc当地 tagtgc当地 aaaacgttgc acatccctcg
 gccglocal gattt aatcgc当地 atcgactcaa gtgatc当地
 tggttctt gtattcccgat gtc当地 cgtc当地 ggc当地
 gccatcttgc当地 aagttagttt cattt当地 atgc当地
 tatgtatcgc acgtcaagaa ttaacaatgc gccc当地
 acgactatga tagagatcaa ataaacgc当地 aattt当地
 aacgtgc当地 atctgtgc当地 gcttcccgat acgacttgc当地
 gttttacga agcgatgaca tgaccccgat agtgc当地
 aaagaactgc cgactacaaa attacccgat atgtcg当地
 attaagccat ccaatcgacc gttagtc当地 tcaggaccgc
 agccgc当地 agtgc当地 gcatc当地 acgtgtgg
 agagcgtcat gtttagacaa gaaagctaca tattt当地
 ttttattgtt aaattgaccc taactccata cacggtattc
 ggggttggg caaaatttcc ggactcgat tgtacatg
 cc当地 cacta ttaatgaaat taaaaattcc aattt当地
 gagaaacatt tggatgaaat aatgc当地 aggaaagaaa
 acatgctgaa caacaagatt aatatgc当地 cgtgtata
 aacgatttga aagaaaacaa tgc当地 cgc当地
 gtttatacta aactgttaca ttgc当地
 aaaaccgatg ttaatc当地 gctctgacgc
 aagtgtgtgg gtgaagtc当地 gcatcttta
 taaaccacca aactgc当地 aaatgaaaac
 ttgc当地 ggatggg
 aaacaattat aaatgc当地 tttgtt
 gcaacaagaa cattt当地 tagtattt
 atcgctgagg taatattaa aatcattt
 gcgacaatatt aattt当地 tcacata
 tcttgc当地 ctttctt
 gtttattatcg tatccatata tttt
 tggctatccatata
 atatgtct
 atagtttgc当地 tgtaattt
 gtgtgttgc当地 ttaattt
 cgggtt
 acgc当地
 acataactt
 tccctt
 atactatt
 cagccatt
 aatgagacgc
 acaact
 atcaca
 ggaaatgtct

4/254

FIGURE 1 (Cont'd)

ctgtcccgat ttatttggaaa cactacaaat taaaggcgag ctttcgtacc
 aacttgttag caatatttatt agacagctgt gtgaagcgct caacgatttg
 cacaaggcaca atttcataca caacgacata aaactcgaaa atgtcttata
 tttcgaagca cttgatcgcg tgtatgtttg cgattacgga ttgtgcaaac
 acgaaaaactc acttagcgta cacgacggca cggtggagta ttttagtccg
 gaaaaaaaaattc gacacacaac tatgcacgtt tcggttact ggtacgcggc
 gtgttaacat acaagttgct aacgtaatca tggtcatagc tgtttcgtt
 gtgaaattgt tattccgtca caattccaca caacatacga gccggaaagca
 taaagtgtaa agcctgggg gcctaattgag tgagctaact cacattaatt
 gcgttgcgct cactgcccgc tttccagtcg ggaaacctgt cgtgccagct
 gcattaatga atcggccaac gcgcggggag aggccgttgc cgtattggc
 gctcttccgc ttccctcgctc actgactcg tgcgcgtcggt cggtcggt
 cggcgagcgg tattcagtc a c t c a a a g g c g t t a a t a c g g t t a t c c a c a g a
 atcaggggat aacgcaggaa agaacatgtg agcaaaaaggc cagaaaaagg
 ccaggaacccg taaaaaggcc gcgttgcgttgc cgttttcca taggctccgc
 cccccctgacg agcatcacaa aaatcgacgc tcaagtcaga ggtggcgaaa
 cccgacagga ctataaagat accaggcggt tccccctgga agtccttcg
 tgcgcgtctcc tttcccgacc ctggcgctta ccggataacct gtccgcctt
 ctcccccttcgg gaagcgtggc gcttctcat agtcacgct gtaggtatct
 cagttcgggtg tagtcgttc gcttcaagct gggctgtgtg cacgaacccc
 ccgttcagcc cgaccgctgc gccttataccg gtaactatcg tctttagtcc
 aaccccgtaa
 gacacgactt atcgcactg gcagcagccca ctggtaacag gattagcaga
 gcgaggtatg taggcgggtgc tacagagttc ttgaagtggt ggcctaacta
 cggctacact agaaggacag tatttggtat ctgcgtctg ctgaagccag
 ttaccttcgg aaaaagagtt gtagctctt gatccggcaa acaaaccacc
 gctggtagcg gtggttttt tttttgcag cagcagatta cgcgcagaaa
 aaaaggatct caagaagatc ctttgatctt ttctacgggg tctgacgctc
 agtggaaacga aaactcacgt taaggattt tggcatgag attatcaaaa
 aggatcttca cctagatcct tttaaattaa aatgaagtt ttaaatcaat
 ctaaaagtata tatgagtaaa cttggctcga cagttaccaa tgcttaatca
 gtgaggcacc tatctcagcg atctgtctat ttcttcatc catagttgcc
 tgactccccg tcgtgttagat aactacgata cgggagggtt taccatctgg
 cccccagtgt gcaatgatac cgcgcagacc acgctcaccg gctccagatt
 tatcagcaat aaaccagcca gcccggaaaggcc cgcgcgcag aagtggctt
 gcaactttat ccgcctccat ccagtctatt aattttgcgc aacgttgc ggcattgcta
 agtaagtatg tcgcccgtt atagttgcgc caacgttgc ggcattgcta
 caggcatcgt ggtgtcacgc tcgtcgttt gtagggcttc attcagctcc
 ggttcccaac gatcaaggcg agtacatga tcccccatgt tttttttttt
 agcggttagc tccttcggc ctcgcgttgc tttttttttt tttttttttt
 cagtgttattc actcatgggtt atggcagcac tgcataattt tttttttttt
 atgccatccg taagatgttt ttctgtgact ggtgagtttcaaccaagtc
 attctgagaa tagtgtatgc ggcgcaccgag ttgcctttgc cccggcgtcaa
 tacggataaa taccgcgcac catagcagaa cttttttttt tttttttttt
 gaaaaacgtt cttcggggcg aaaactctca aggatcttac cgctgtttag
 atccagttcg atgtaaccca ctcgtgcacc caactgatct tcagcatctt
 ttactttcac cagcggttgc gggtagccaa aaacagggaa gaaaaatgcc
 gaaaaaaaaagg gaataaggc gacacggaaa ttgttgcatac tcataactt
 cttttttca tattattgaa gcatttatca gggttattgt ctcatgagcg
 gatacatatt tgaatgtatt tagaaaaata aacaaatagg gttccgcgc
 acatttcccc gaaaagtgc acctgacgctc taagaaacca ttattatcat
 gacattaacc tataaaaaata ggcgtatcac gaggccctt cgtctcgcc
 gtttcgggtga tgacgggtgaa aacctctgac acatgcagct cccggagacg
 gtcacagctt gtctgttaagc ggtatgcggg agcagacaag cccgtcaggg

5/254

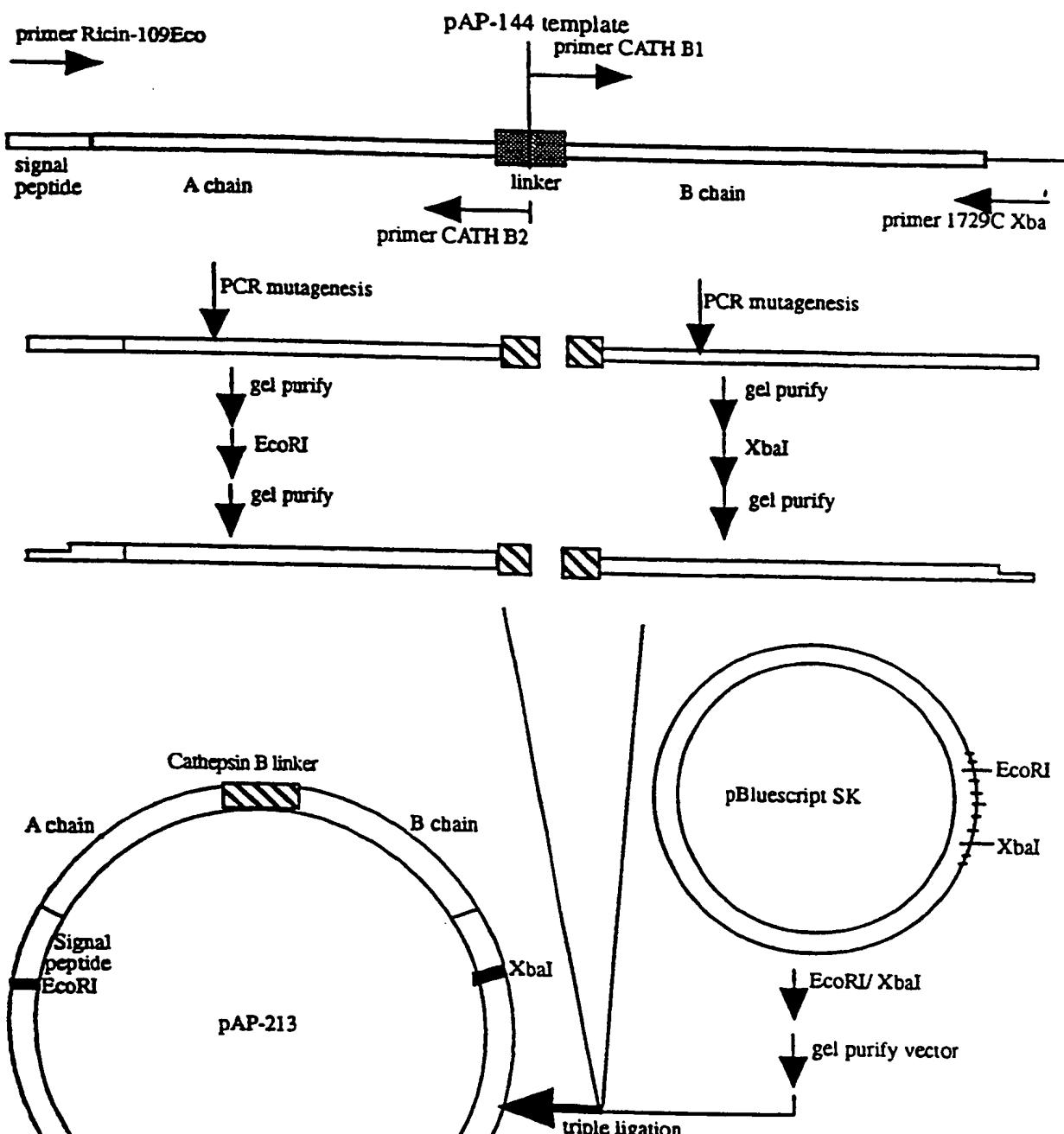
FIGURE 1 (Cont'd)

atcaaataat agttgctgat atcatggaga taattaaaat gataaccatc
tcgcaaataa ataagtattt tactgtttc gtaacagtt tgtaataaaaa
aaacctataa atattccgga ttattcatac cgtcccacca tcgggcgcgg
atccccggta ccttctagaa ttccggagcg gccgctgcag atctgatcct
ttccctgggac ccggcaagaa ccaaaaactc actctttca aggaaatccg
taatgttaaa cccgacacga tgaagcttg cgttggatgg aaaggaaaaag
agttctacag ggaaacttgg acccgcttca tggaaagacag cttccccatt
gttaacgacc aagaagtgat ggatgtttc cttgttgtca acatgcgtcc
cantagaccc aaccgttgtt acaaattcct ggcccaacac gctctgcgtt
gcgaccccca ctatgtaccc catgacgtga ttaggatcgt cgagccttca
tgggtgggca gcaacaacga gtacccgatc agcctggcta agaaggcgg
cggctgcccataatgaacc ttcactctga gtacaccaac tcgttcgaac
agttcatcga tcgtgtcatc tgggagaact tctacaagcc catcgtttac
atcggtaccc actctgtga agaggaggaa attctccttg aagttttcc
ggtgttcaaa gtaaaggagt ttgacccaga cgcacccctg ttcactggc
cggcgttatta aaacacgata cattgttatt agtacatttta ttaagcgcta
gattctgtgc gttgttatt tacagacaat tgggtacgt attttataa
ttcatttaat ttataatctt tagggtggta tggtagagcg aaaatcaa
gattttcagc gtcttatata ctgaatttta atattaaatc ctcaatagat
ttgtaaaata ggttgcattt agttcaaac aagggttgtt ttccgaacc
gatggctgga ctatctaattt gatttcgct caacgccaca aaacttgcca
aatcttgttag cagaatcta gcttgcga tattgttgc tgggttgc
tgtaataaaag gttcgacgtc gttcaaaata ttatgcgtt ttgtatttct
ttcatcactg tcgttagtgc acaattgact cgacgttaac acgttaaaa
aagcttggac atatttaaca tcggcgtgt tagctttatt
tcgtcgctgt cccaaaccctc gtcgttagaa gttgttccg aagacgattt
tgccatagcc acacgacgccc tattaaattgt gtcggcta acgtccgcga
tcaaatttgt agttgagct tttggaatta tttctgattt cgggcgtttt
tgggccccgtt tcaatctaac tggcccgat ttaatttcg acaacacgtt
agaaaagcgt ggtgcaggcg gttgttaacat ttacagacggc aaatctacta
atggcggccg tgggtggagct gatgataaat ctaccatcg tggaggcga
ggcggggctg gcggcggagg cggaggcgga ggtggtggcg gtatgcaga
cgccgggtta ggctcaaattt tcttttagg caacacagtc ggcaccccaa
ctattgtact gtttcgggc gccgttttgc gtttgcacggc tctgagacga
gtgcgatttt tttcgattt aatagcttcc aacaattgtt gtctgtcg
taaaggtgca gcgggtttagt gttccgtcg cattgggtggaa gcggccggca
attcagacat cgatgggtgt ggtgggtgtt gaggcgctgg aatgttaggc
acgggagaag gtgggtggccg cggtgccgccc ggtataattt gttctgggtt
agtttgcg cgcacgattt tgggcaccgg cgcaggcgcc gctggctgca
caacggaaagg tcgtctgctt cgaggcagcg cttgggggtgg tggcaattca
atattataat tggaaatacaa atcgtaaaaaa tctgtataa gcattgtaat
ttcgctatcg ttaccgtgc cgatatttaa caaccgctca atgtaagcaa
ttgtatttta aagagattt ctcaagctcg cccgacgccc ataacaagcc
ttttcatttt tactacaga ttgttagtggc gagacactt cgtgtcg
acgtacatgt atgctttgtt gtcaaaaacg tcgttggcaa gctttaaaat
attttaaaaga acatctctgt tcagcaccac tgggttgtcg taaatgttgc
ttttgataat ttgcgtttcc gcagtatcg cacgttcaaa aaattgtatgc
gcattcaattt tgggtttctt attattgaat aaataagatt gtacagattt
atattctacga ttgcgtcatgg ccaccacaaa tgctacgctg caaacgctgg
tacaattttt cggaaaactgc aaaaacgtca aaactcggtt taaaatataatc
aacggggcgtt tggcaaaaat atctattttt tcgcacaagc ccactagcaa
attgtattttg cagaaaacaa tttcgccgca caattttac gtcgacgaaa
taaaagtca ccagttaatg agcgaccacc caaattttat aaaaatctat
ttaatcacg gttccatcaa caaccaagtg atcgtgatgg actacatttg

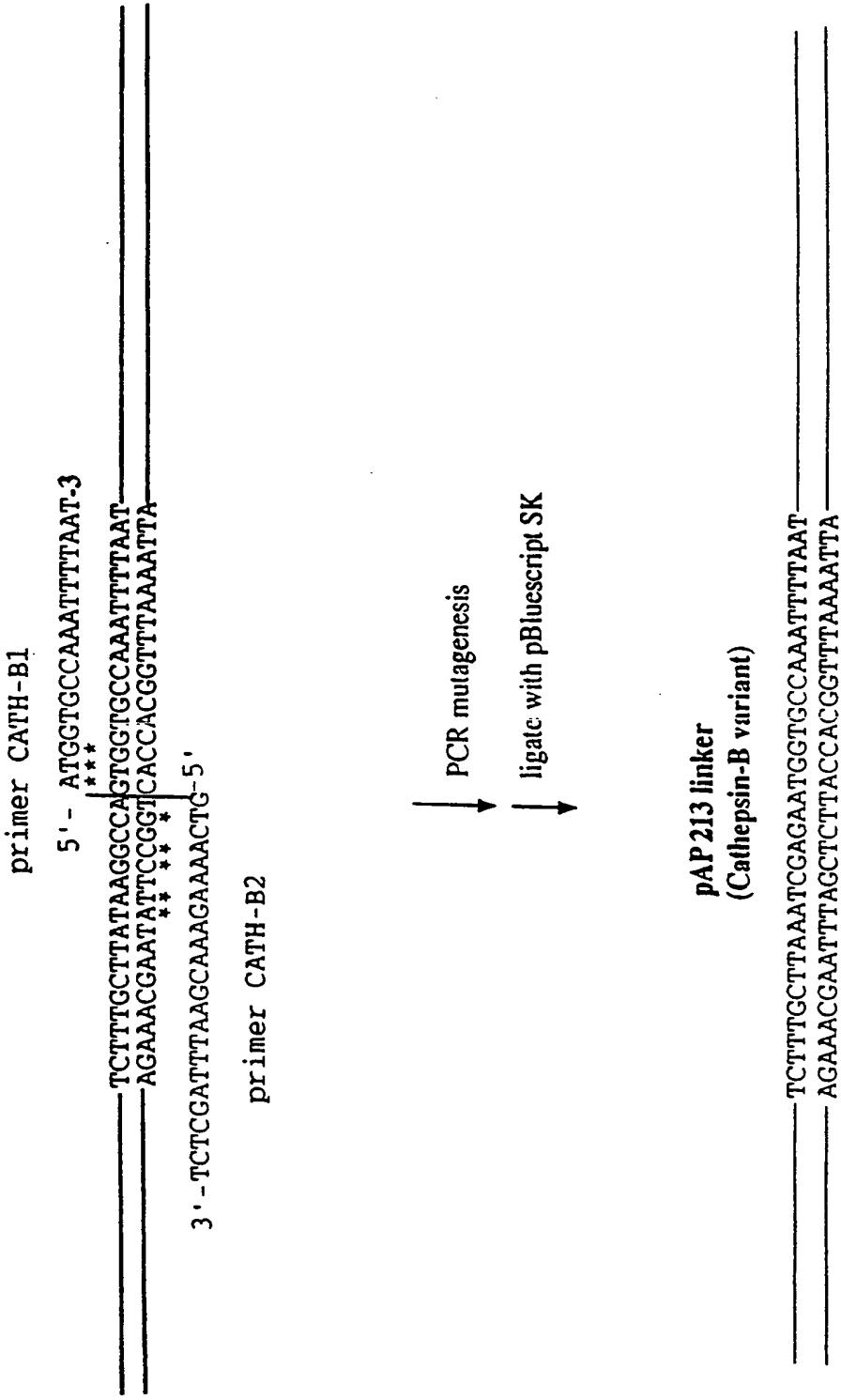
FIGURE 1 (Cont'd)

6/254

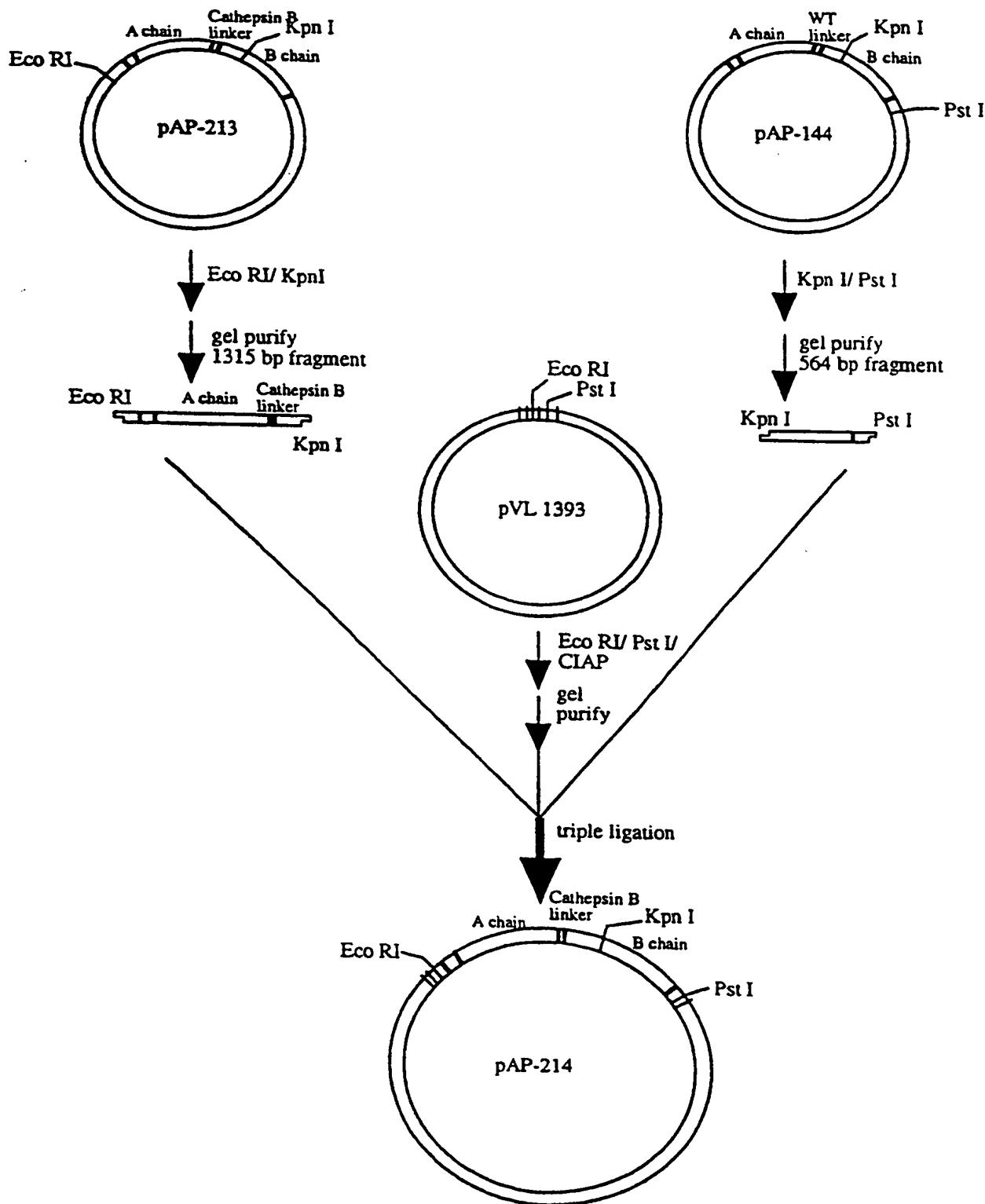
7/254

FIGURE 2A

8/254

FIGURE 2B**WT preprorcin linker**

9/254

FIGURE 2C

10/254

FIGURE 2D

10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAATACTATTGTAATATGGATGTATGCAGT			
	CTTAAGTACTTTGCCCTCTTATGATAACATTATACCTACATACGTCA			
51	GGCAACATGGCTTGTGATCCACCTCAGGGTGGTCCTTCACATTAG			
	CCGTTGTACCGAAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAA			
101	AGGATAACAACATATTCCCCAACAAACACCAATTATAAACTTACCA			
	TCCTATTGTTGATAAGGGTTGTTATGGGTTAATATTGAAATGGTGT			
151	GCGGGTGCCTACTGTCAAAGCTACACAAACTTTATCAGAGCTGTCGCC			
	CGCCCCACGGTGACACGTTGATGTGTTGAAATAGTCTGACAAGCGCC			
201	TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAAGTGTGCCA			
	AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT			
251	ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACCTCTCA			
	TGTCTCAACCAAACGGATATTGGTTGCCAATAAAATCAACTTGAGAGT			
301	AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA			
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT			
351	TGTGGTCGGCTACCGTGTGAAATAGCGCATATTCTTCATCCTGACA			
	ACACCCAGCGATGGCACGCCACCTTATCGCGTATAAAGAAAGTAGGACTGT			
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTCACTGATGTTCAAAAT			
	TAGTCCTTCTACGTCTCGTTAGTAGAAAGTAGACTACAAGTTTA			
451	CGATATACATGCCATTGGTGGTAATTATGATAGACTGAAACAACTTGC			
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG			
501	TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG			
	ACCATTAGACTCTCTTTATAGCTAACCCCTTACCGGTGATCTCCTCC			
551	CTATCTCAGCGCTTTATTACAGTACTGGTGGCACTCAGCTTCAACT			
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA			
601	CTGGCTCGTCCCTTATAATTGATCCAATGATTCAAGCAGCAAG			
	GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTCGTT			
651	ATTCCAATATATTGAGGGAGAAATCGCAGCAGAGAATTAGGTACAACCGGA			
	TAAGGTTATATAACTCCCTCTTACCGCGTGTCTTAATCCATGTTGGCCT			
701	GATCTGCACCAAGATCCTAGCGTAATTACACTTGAGAAATGTTGGGGGAGA			
	CTAGACGTGGCTAGGATCGCATTATGTGAACTCTTATCAACCCCTCT			
751	CTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT			
	GAAAGGTGACGTTAAGTCTCAGATTGGTCTCGGAAACGATCAGGTTA			
801	TCAACTGCAAAGACGTAATGGTCCAATTCACTGATGTGAGTA			
	AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT			
851	TATTAATCCCTATCATAGCTCTCATGGTGTAGATGCGCACCTCCACCA			
	ATAATTAGGGATAGTATCGAGAGTACCCACATATCTACCGTGAGGTGGT			
901	TCGTCACAGTTCTTGCTTAAATCGAGAATGGTCCAAATTAAATGCA			
	AGCAGTGTCAAAGAAACGAATTAGCTCTTACACCGTTAAAATTACG			

11/254

FIGURE 2D (CONT'D)

951 TGATGTTGTATGGATCCTGAGCCCATACTGCGTATCGTAGGTCGAAATG
 ACTACAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC

 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACTACCCCTACCTTCTAAGGTGTTGCCCTTGCGTTAT

 1051 CAGTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTAGATTATGTCTACGTTAGTCGAGACCTGAAA

 1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
 CTTTTCTCTGTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC

 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAAACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGGACGT

 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCCATTACCTGGTAGTATTTAGG

 1251 CAGATCTAGTCAGTTAGCAGCGACATCAGGGAACAGTGGTACCCACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTGTCAACCATGGTGTG

 1301 TTACAGTCAAACCAACATTATGCCGTTAGTCAGGTTGGCTTCCTACT
 AATGTCACGTTGGTTGTAATACGGCAATCAGTCCAACCGAAGGATGA

 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGCTGTG
 TTATTATGTGTTGGAAAACAATGTTGTAACAACCCGATATACCAAGACAC

 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTGTTATCACCTGTCATACCTATCCCTGACATCGTCACCTT

 1451 AGGCTGAACAAACAGTGGGCTCTTATGCAAGATGGTTCAATACGTCCCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

 1501 CAAAACCGAGATAATTGCCCTACAAGTGAATTCTAATATACGGAAACAGT
 GTTTGGCTCTATTAACGGAATGTTCACTAAGATTATGCCCTTGTC

 1551 TGTTAAGATCCTCTTGTGGCCCTGCATCCTCTGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTTAGGAGACCGGTTGCTACCTACA

 1601 TCAAGAATGATGGAACCATTAAATTGTTAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTGGTAAATTAAACATATCACCTAACCAACATCTA

 1651 GTGAGGCAGTCGGATCCGAGCCTAAACAAATCATTCTTACCCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT

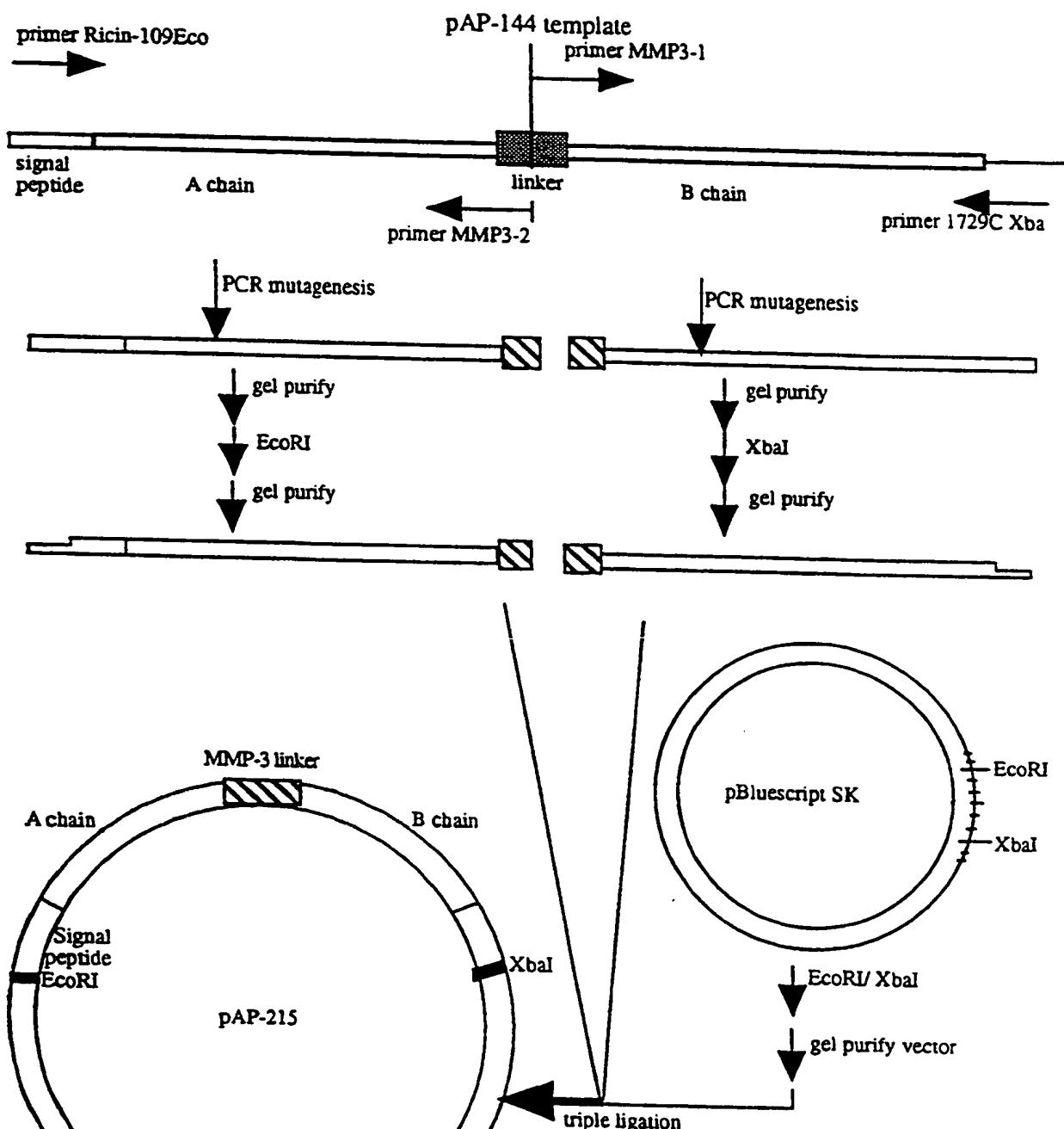
 1701 TGGTGACCCAAACCAAATATGGTTACCAATTATTGATAGACAGATTACT
 ACCACTGGGTTGGTTATACCAATGGTAATAAAACTATCTGTCTAATGA

 1751 CTCTTGAGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTTT

 1801 GGACATTGTAATTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAACATTGACTTCCTGTCGTTCAATATAGCTTAAGG

 1851 TGCAG
 ACGTC

12/254

FIGURE 3A**SUBSTITUTE SHEET (RULE 26)**

13 / 254

FIGURE 3B

WT preprorocin linker

primer MMP3-1

5' - TTTTTGGACTTATGAATGCTGATGTTGT - 3'
 * * * * * * * *
 TCTTGCTTATAAGGCCAGTGGTGCCTAAATTAAAT
 AGAACGAAATATTCGGTACCCACGGTTAAAATA
 * * * * * * * *
 3' - GGTAGCACTGTCAAAGCAGGGCTTCGGTGTCTT - 5'

primer MMP3-2

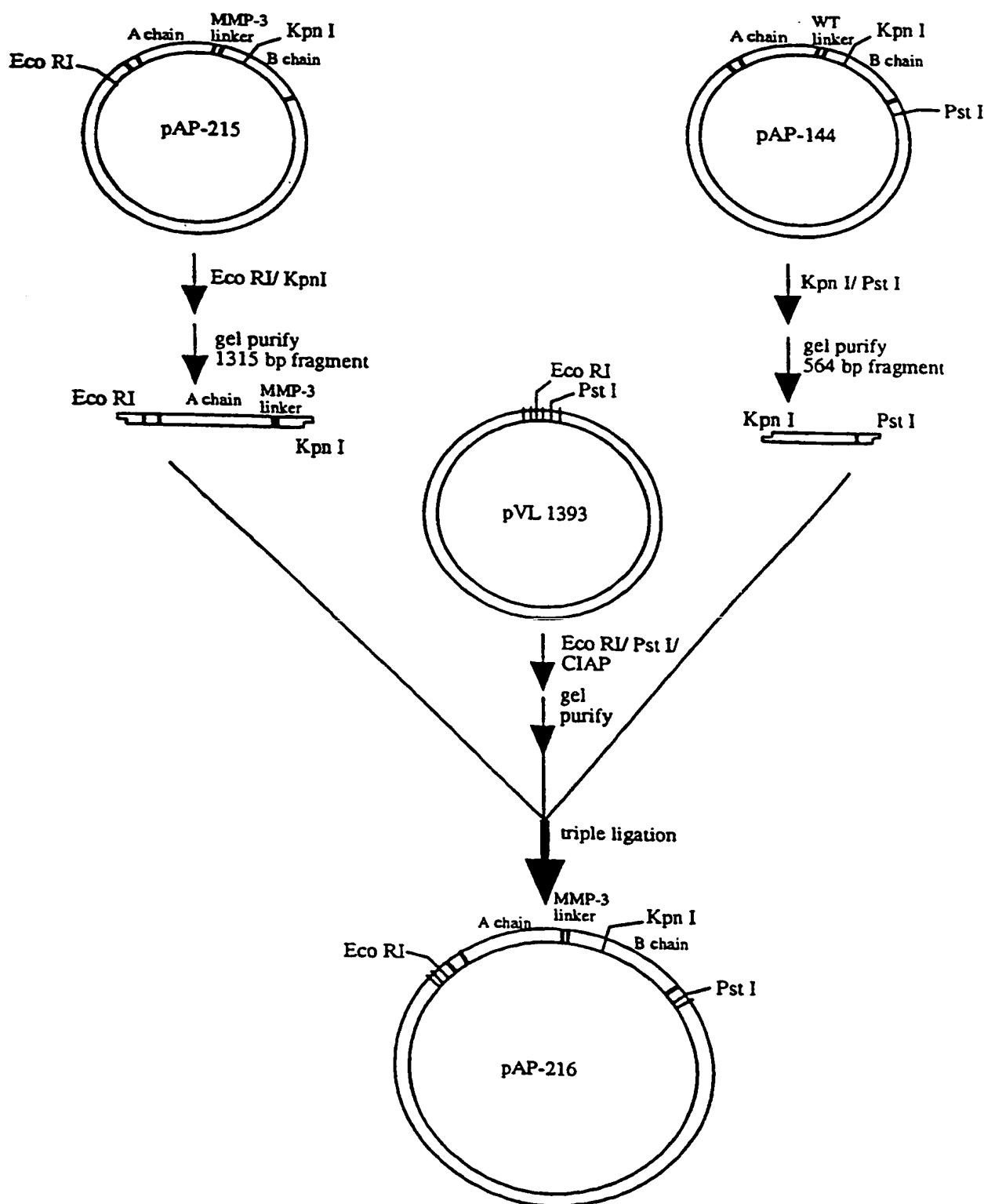
PCR mutagenesis

Ligate with pBluescript SK

pAP215 linker (MMP-3 variant)

--CGTCCGAAGCCACAGCAATTTTGGACTTATGAAATGCAGGGCTCGGTGTCAAAAAACCTGAATACCTTA-

14/254

FIGURE 3C

SUBSTITUTE SHEET (RULE 26)

15/254

FIGURE 3D

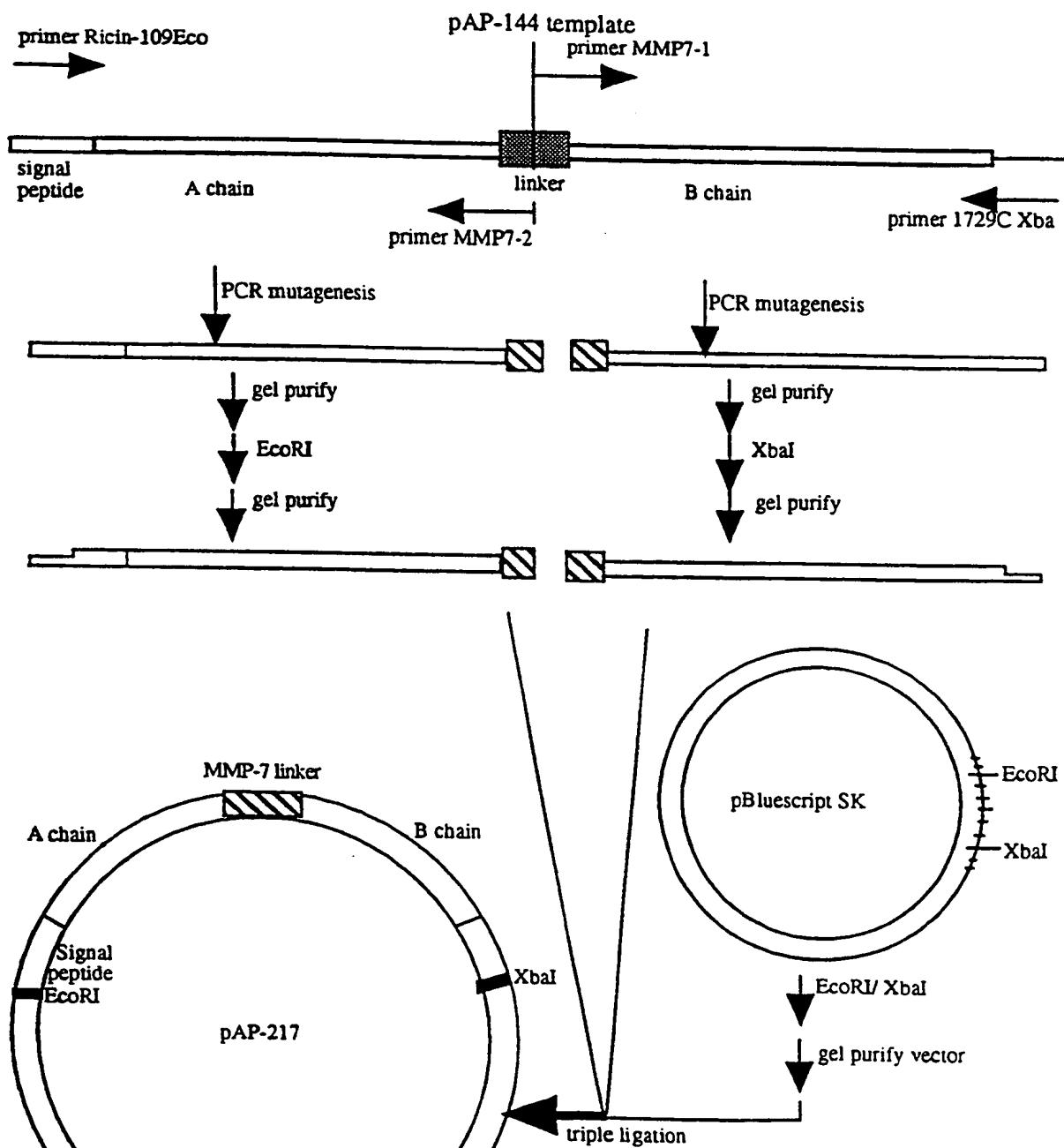
10	20	30	40	50
1 GAATTCA	TTGAAACCGGGAGGAA	ATCTATTGTA	ATATGGATGT	TATGCAGT
	CTTAAGTACTT	GGCCCTCCTT	TATGATAAC	ATTACCTACATACGTCA
51 GGCAACATGG	CTTGTGTTGGATCC	CACCTCAGGGTGG	TTCACATTAG	
	CCGTTGTA	CGAACAAA	ACCTAGGTGGAGT	CCCACCAGAAAAGTGTAA
101 AGGATAACA	ACATATTCCCCA	AAACAATACCCAA	TTAAACTTACCA	ACA
	TCCTATTGTTG	TATAAGGGTTG	TATGGGTTAATATTG	AAATGGTGT
151 GCGGGTGC	CACTGTGCAAAGCT	ACACAAACTT	TATCAGAGCTG	TTCGCCG
	CGCCCACGGTGAC	CGTTCGATG	TGTTGA	AAATAGTCTCGACAAGCGCC
201 TCGTTAACAA	ACTGGAGCTGATG	TGAGACATG	ATATACCA	AGTGTGCCAA
	AGCAAATTGTTG	ACACTCG	ACTAC	TGTACTATATGGTCACAACGGTT
251 ACAGAGTTGG	TTGCCTATAAA	ACCAACGGTT	TTAGTTAG	TGAACTCTCA
	TGTCTCAACCA	ACGGATA	TTGGTGC	AAATAAAATCAACTTGAGAGT
301 AATCATGCAGAG	CTTCTGTTACAT	AGCGCTGGATG	TCACCAATGC	CATA
	TTAGTACGTCTC	GAAAGACA	ATGTA	TCGCGACCTACAGTGGTTACGTAT
351 TGTGGTCGG	CTACCGTGCTGG	AAATAGCGC	ATATTC	TTCATCCTGACA
	ACACCAGCCGATGG	CACGAC	CTTATCGCGT	ATAAGAAAGTAGGACTGT
401 ATCAGGAAGATG	CAGAAGCA	ATCACTCAT	CTTCACTGATG	TGTCAAAAT
	TAGTCCTCTACG	TCTCGTTAGT	GAGTAGAA	AGTAGACTACAAGTTTA
451 CGATATA	CATTGCG	CTTGGTGG	ATTATGATAG	ACTTGAACAACTTG
	GCTATATGTAAGCG	AAACCACCA	TTAAACTATCTG	AACCTGTTGAACG
501 TGGTAATCTGAGAG	AAAATATCGAG	TTGGGAAATGGT	CCACTAGAGGAGG	
	ACCATTAGACTCT	TTTATAGCT	CAACCC	TTTACCAAGGTGATCTCCTCC
551 CTATCTCAGCG	CTTATTACAGT	ACTGGTGG	CACTCAGCT	TCCA
	GATAGAGTCG	CGAAATAA	ATATGT	CATGACCACCGTGAGTCGAAGGTTGA
601 CTGGCTGTT	CTTATAATTG	CATCCA	ATGATTTCAGAAGCAGCAAG	
	GACCGAGCAAGG	AAATATTAA	ACGTAGGTT	ACTAAAGTCTCGTCGTT
651 ATTCCAATATATTGAGG	GAGAAATGCG	CACGAGA	ATTAGGTAC	AAACCGGA
	TAAGGTTATATA	ACTCC	CTTACCGCGT	GCTCTTAATCCATGTTGGCCT
701 GATCTGCACCA	GATCCTAGCG	TAATTAC	ACTTGGAGA	ATAGTTGGGGAGA
	CTAGACGTGG	TCTAGGATCG	CATTAA	TGTGAACTCTTATCAACCCCCTCT
751 CTTTCACTGCA	ATTCAAGAGT	CTAACCA	AGGAGC	CTTGCTAGTCCAAT
	GAAAGGTGACG	TTAAGT	TCTCAGATTGGT	CCCTCGGAAACGATCAGGTTA
801 TCAACTGCA	AAAGACGT	AAATGGT	CCAAATT	CAGTGTGAGTA
	AGTTGACG	TCTGCATTACCA	AGGTTAAGT	CACACATGCTACACTCAT
851 TATTAATCC	CTATCATAGCT	CTCATGGTGT	ATAGATG	CGCACCTCCACCA
	ATAATTAGGG	ATAGTATCGAGAGT	ACCA	ATATCTACCGCGTGGAGGTGGT
901 TCGTCACAG	TTCTGCGAAGCC	CACAGCA	ATT	TTGGACTTATGAATGCA
	AGCAGTGT	CAAAGCAGG	CTCGGTG	CTCGTTAAAAACCTGAATAC
951 TGATGTTG	TATGGATCCTG	GAGCCC	CATAGT	CGTAGGTCGAAATG

16/254

FIGURE 3D (CONT'D)

ACTACAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACTACCCCTACCTTAAGGTGTTGCCTTGCGTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTAGATTATGTCAGTTACGTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGATGCA
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCTTACCTGGTAGTATTAGG
 1251 CAGATCTAGTCTAGTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCTTGTACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTATGCCGTTAGTCAAGGTTGGCTTCCTACT
 AATGTCACGTTGGTTGTAATACGCAATCAGTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTTGGAAAACAATGTTGTAACAACCCGATATACAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAA
 GAACGTTGTTATCACCTGTCACCTATCCTGACATCGTCACCTT
 1451 AGGCTGAACAAACAGTGGGCTTTATGCAGATGGTTCAATACGTCCCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCCTACAAGTGATTCTAATATACGGGAAACAGT
 GTTTGGCTCTATTAACGGAATGTTACTAAGATTATATGCCCTTGTCA
 1551 TGTTAAGATCCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTAAATTGTATAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTGGTAAATTAAACATATCACCTAACCAATCTA
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTACCCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAATATGGTACCAATTATTGTATAGACAGATTACT
 ACCACTGGGTTGGTTATACCAATGGAATAAAACTATCTGTCTAATGA
 1751 CTCTTGAGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTT
 1801 GGACATTGTAATTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAACATTGACTTCCCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

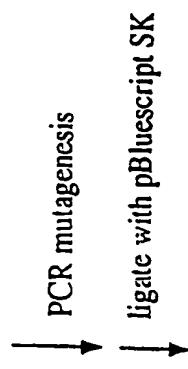
17/254

FIGURE 4A**SUBSTITUTE SHEET (RULE 26)**

18/254

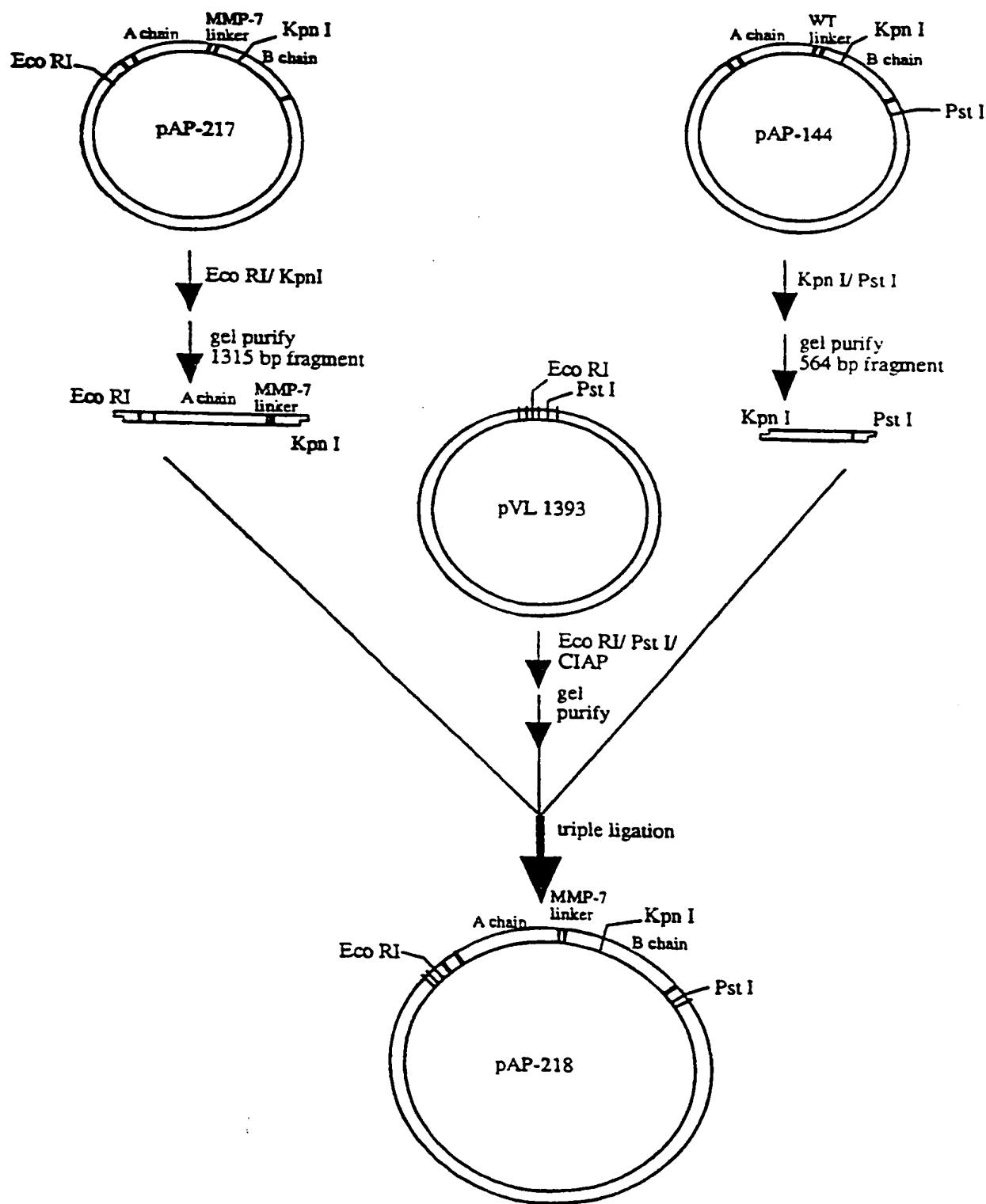
FIGURE 4B**WT preprorin linker****primer MMP7-1**

5' - TGTGGCGAAGTTAAATGCTGATGTT-3'
 * * * *
 TCTTGCTTATAAGGCCAGTGTGCCAAATTAAAT
 AGAACGAAATTCGGTACCCACGGTTAAATTA
 * * * * *
 3' - AGTGTCAAAGAACGCAGGTGACCGT-5'

primer MMP7-2**pAP217 linker
(MMP-7 variant)**

TCTTGCGTCCACTGGCATTTGGCGAACGTTTAAAT
 AGAACGCAAGGTGACCGTAAACCCGCTTCAAAATTA

19/254

FIGURE 4C

SUBSTITUTE SHEET (RULE 26)

20/254

FIGURE 4D

10 20 30 40 50

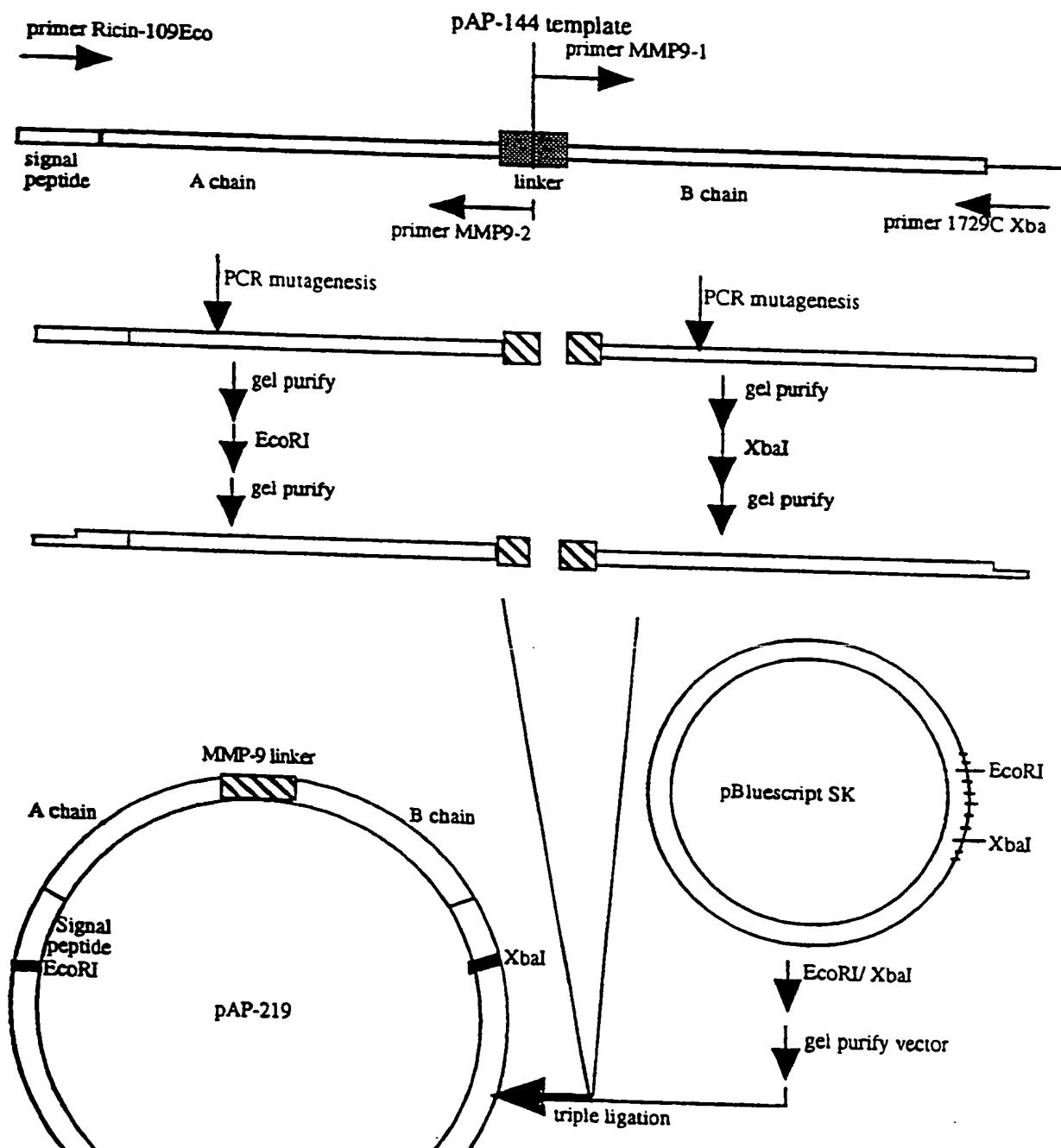
1 GAATTCATGAAACGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
 CTTAAGTACTTTGGCCCTCCTTATGATAACATTATAACCTACATACGTCA
 51 GGCAACATGGCTTGTGATCCACCTCAGGGTGGCTTTCACATTAG
 CGGTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC
 101 AGGATAACAACATATTCCCCAAACAAATACCCAATTATAAACCTTACCA
 TCCTATTGTTGTATAAGGGTTGTATGGGTAAATATTGAAATGGTGT
 151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTCGCG
 CGCCCACGGTGACACGTTCGATGTGTTGAAATAGTCTCGACAAGCGCC
 201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCACTGTGTTGCCAA
 AGCAAATTGTTGACCTCGACTACACTGTACTATATGGTCACAACGGTT
 251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACCTCTCA
 TGTCTCAACCAAACGGATATTGGTTGCCAAATAAAATCAACTTGAGAGT
 301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
 351 TGTGGTCGGCTACCGTGTGGAAATAGCGCATATTCTTCATCCTGACA
 ACACCAGCCATGGCACGACCTTATCGCGTATAAAGAAAGTAGGACTGT
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTCACTGATGTTCAAAAT
 TAGTCTTCTACGTCTCGTTAGTAGAAAGTAGTACTACAAGTTTA
 451 CGATATACTCGCCTTGGTGGTAATTATGATAGACTTGAACAACCTGC
 GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG
 ACCATTAGACTCTCTTATAGCTAACCCCTTACCAAGGTGATCTCCTCC
 551 CTATCTCAGCGCTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
 GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
 601 CTGGCTCGTCTTATAATTGATCCAAATGATTTCAGAAGCAGCAAG
 GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTGTTC
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
 TAAGGTTATATACTCCCTTTACCGCTGCTTAAATCCATGTTGGCCT
 701 GATCTGCAACAGATCCTAGCGTAATTACACTTGGAGAATAGTTGGGGAGA
 CTAGACGTGGCTAGGATCGCATTAAATGTAACCTTATCAACCCCTCT
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT
 GAAAGGTGACGTTAAGTCTCAGATTGGTCTCGGAAACGATCAGGTTA
 801 TCAACTGCAAAGACGTAATGGTCAAATTCAAGGTTAAGTCACACATGCTACACTCAT
 AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCCACATATCTACCGTGGAGGTGGT
 901 TCGTCACAGTTCTTGGCGTCCACTGGCATTGTGGCGAAGTTTAATGC
 AGCAGTGTCAAAGAAACGCAGGTGACCGTAAACCCGCTTCAAAATTACG
 951 TGATGTTGTATGGATCCTGAGCCATAGTGCCTAGTGTAGGTCGAAATG

21/254

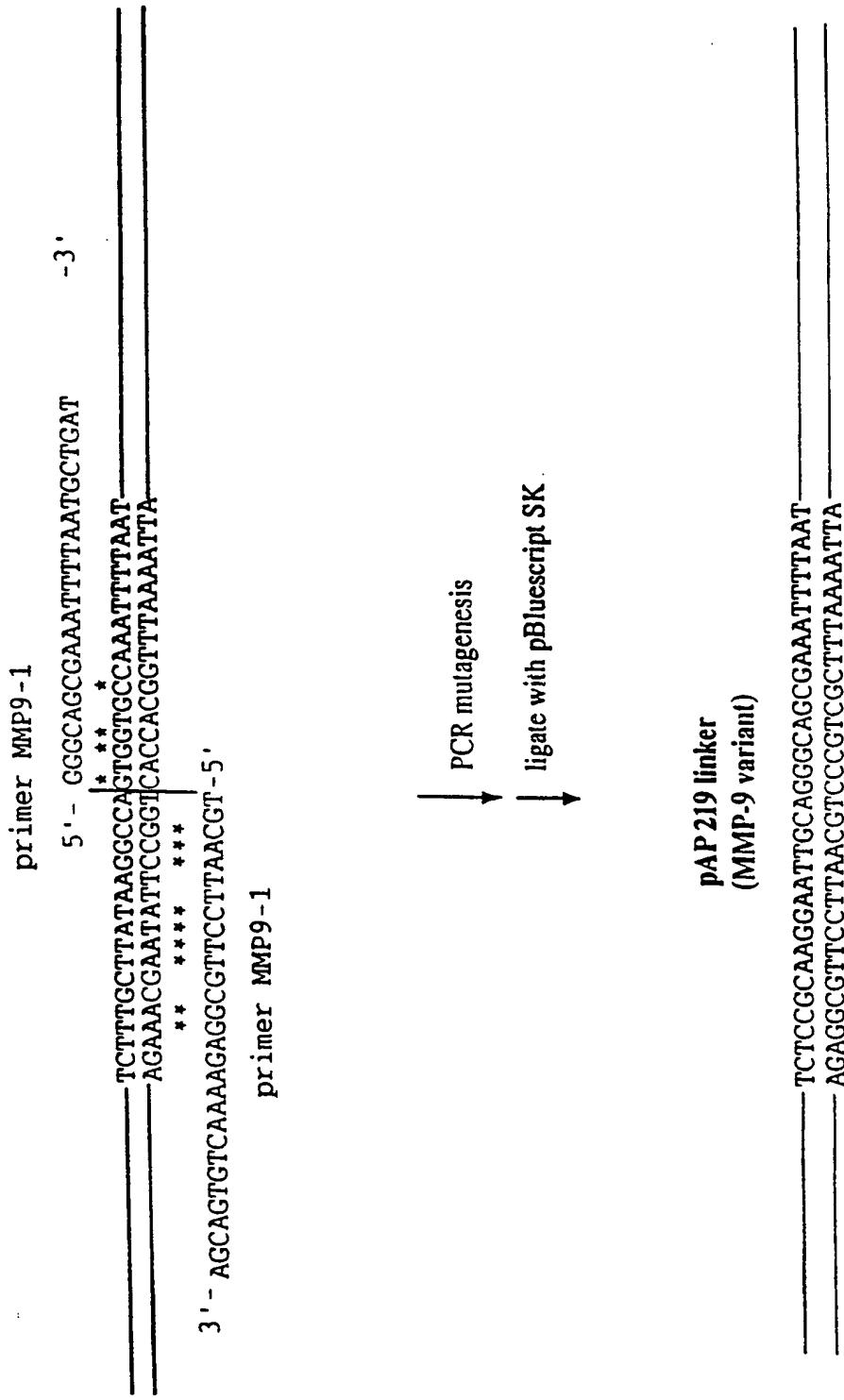
FIGURE 4D (CONT'D)

ACTACAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACTACCTTACCTTCAAGGTGTTGCCCTTGCCTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATAACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTAGATTATGTCTACGTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAATGGAAGTGTTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCCATTACCTGGTAGTATTAGG
 1251 CAGATCTAGTCTAGTTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATAAAATCGTCGCTGTAGTCCTGTACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTATGCGCTTAGTCAGGTTGGCTTCCTACT
 AATGTCACGTTGGTTGTAATAACGCAATCAGTCCAACCGAAGGATGA
 1351 AATAATACACAAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTTGGAAAACAATGTTGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTGTTATCACCTGTCATAACCTATCTCCTGACATCGTCACTTT
 1451 AGGCTGAACAAACAGTGGGCTTTATGCAAGTGGTTCAATACGTCCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCTTACAAGTGTGATTCTAATATAACGGAAACAGT
 GTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTGTCA
 1551 TGTTAAAGATCCTCTCTGTTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAAATGATGGAACCATTAAATTGTATAGTGGATTGGTAGAT
 AGTTCTTACTACCTTGGTAAAATTAAACATATCACCTAACCAACATCTA
 1651 GTGAGGCGATCGGATCCGAGCCTAAACAAATCATTCTTACCCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGAACCAAACCAAAATATGGTTACCATTATTTGATAGACAGATTACT
 ACCACTGGGTTGGTTATACCAATGTAATAAAACTATCTGTCTAATGA
 1751 CTCTTGCACTGTGTTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTTT
 1801 GGACATTGTAATTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAAACATTGACTTCCCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

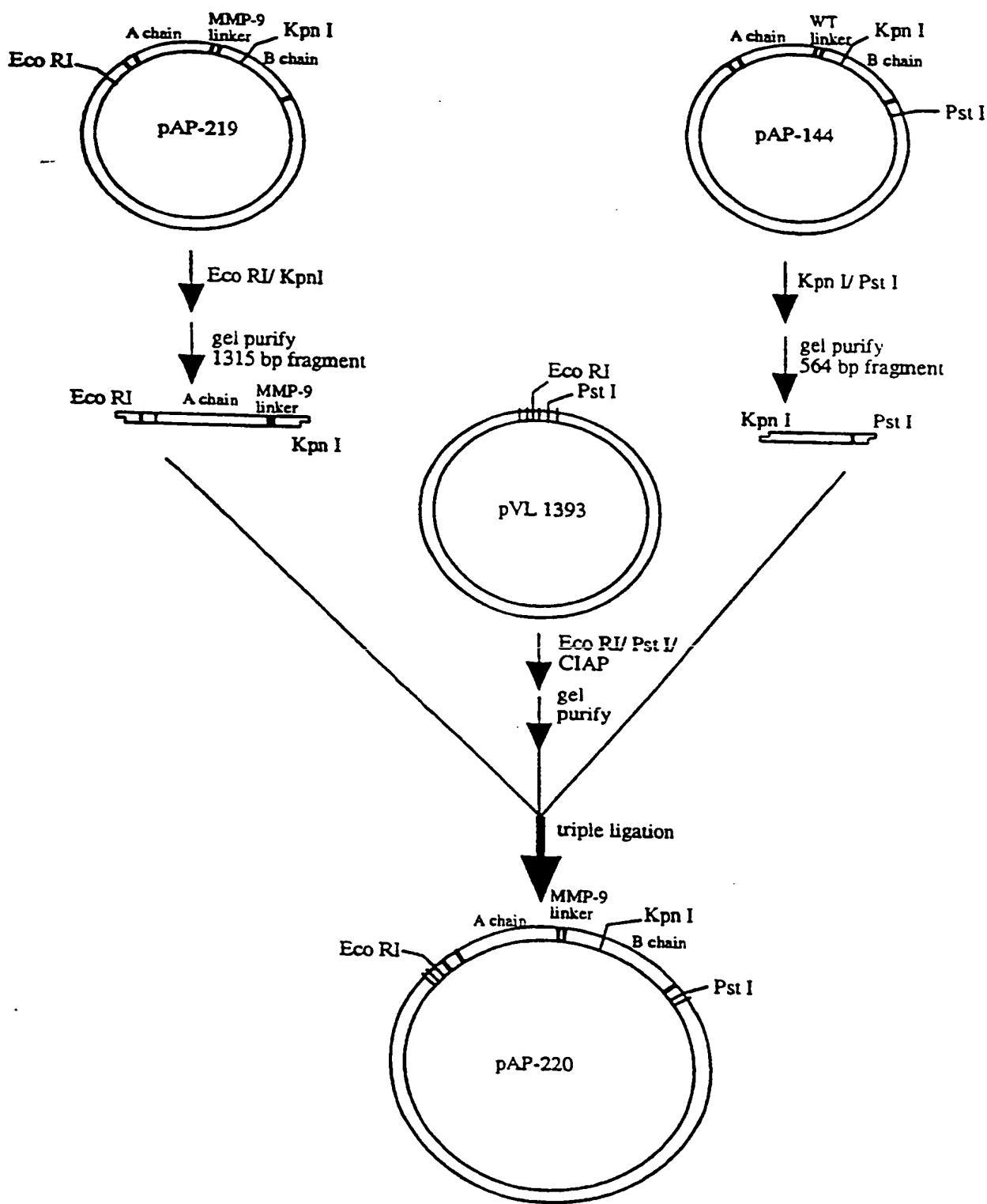
22/254

FIGURE 5A**SUBSTITUTE SHEET (RULE 26)**

23/254

FIGURE 5B**WT preprotecin linker****SUBSTITUTE SHEET (RULE 26)**

24/254

FIGURE 5C

SUBSTITUTE SHEET (RULE 26)

25/254

FIGURE 5D

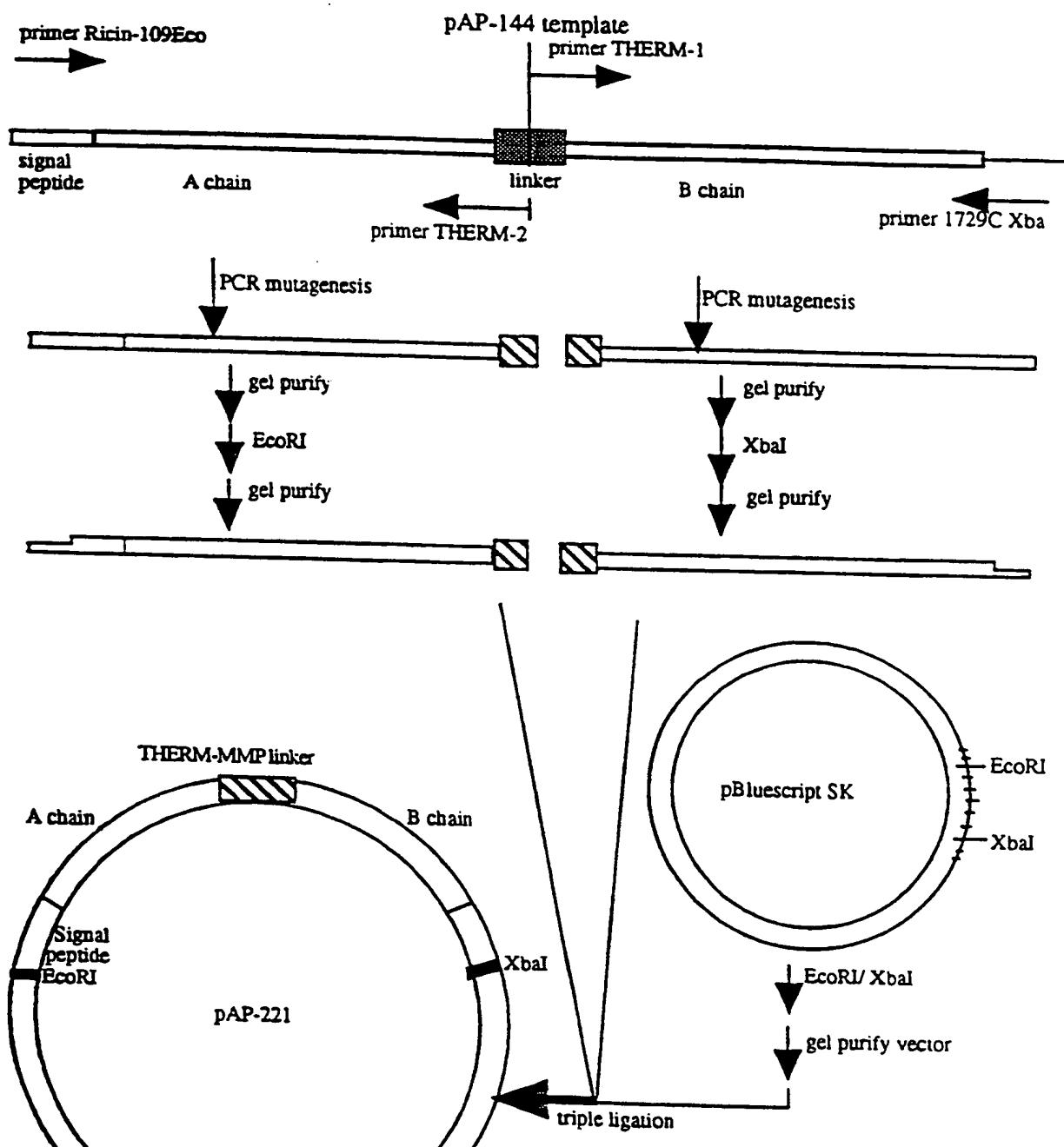
10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAAATGGATGTATGCAGT			
	CTTAAGTACTTTGCCCTCTTATGATAACATTACCTACATACGTCA			
51	GGCAACATGGCTTGTTGGATCCACCTCAGGGTGGCTTCACATTAG			
	CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAA			
101	AGGATAACAACATATTCCCCAACAAACATACCCAAATTATAAACTTACCA			
	ACCTATTGTGTATAAGGGTTGTTATGGGTTAATATTGAAATGGTGT			
151	GCGGGTGCCTACTGTGCAAAGCTACACAAACTTATCAGAGCTGTTCGCGG			
	CGCCCACGGTACACGTTGATGTGTTGAAATAGTCTCGACAAGCGCC			
201	TCGTTAACAACTGGAGCTGATGTGAGACATGATATAACCAGTGTGCCAA			
	AGCAAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT			
251	ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACCTCTCA			
	TGTCTCAACCAAACGGATATTGGTTGCCAAATAACTCAACTTGAGAGT			
301	AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTACCAATGCATA			
	TTAGTACGTCTCGAAAGACAATGTAATCGCACCTACAGTGGTTACGTAT			
351	TGTGGTCGGCTACCGTGCTGGAAAATAGCGCATATTCTTCATCCTGACA			
	ACACCAAGCCGATGGCACGACCTTATCGCTATAAAGAAAGTAGGACTGT			
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTCACTGATGTTAAAAAT			
	TAGTCCTTCTACGTCTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTA			
451	CGATATACTCGCTTGGTGGTAATTATGATAGACTTGAACAACTTGC			
	GCTATATGTAAGCGGAAACCAACCATTAATACTATCTGAACCTGGTTGAACG			
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCACTAGAGGAGG			
	ACCATTAGACTCTTTATAGCTCAACCTTACCAAGGTGATCTCTCC			
551	CTATCTCAGCGCTTATTACAGTACTGGTGGCACTCAGCTTCAACT			
	GATAGAGTCGCGAAATAATAATGTCTACGCTGACCAACCGTGAGTCGAAGGTTGA			
601	CTGGCTGTTCTTATAATTGATCCAAATGATTCAAGCAGCAAG			
	GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTCGTT			
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA			
	TAAGGTTATATAACTCCCTTTACCGCTGCTTAAATCCATGTTGGCCT			
701	GATCTGCACCAAGATCCTAGCGTAATTACACTTGAGAAATAGTTGGGGAGA			
	CTAGACGTGGCTAGGATCGCATTATGTGAACCTTATCAACCCCTCT			
751	CTTTCACTGCAATTCAAGAGTCTAACCAAGGAGCTTGCTAGTCAAT			
	GAAAGGTGACGTTAAGTCTCAGATTGGTCTCGGAAACGATCAGGTTA			
801	TCAACTGCAAAGACGTAATGGTCAAATTCACTGAGTACGATGTGAGTA			
	AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT			
851	TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA			
	ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACGCGTGGAGGTTG			
901	TCGTCACAGTTCTCCGCAAGGAATTGCAGGGCAGCGAAATTAAATGC			
	AGCAGTGTCAAAAGAGGGCGTCTTAACTACGCCCCGTCGTTAAAATTACG			

26/254

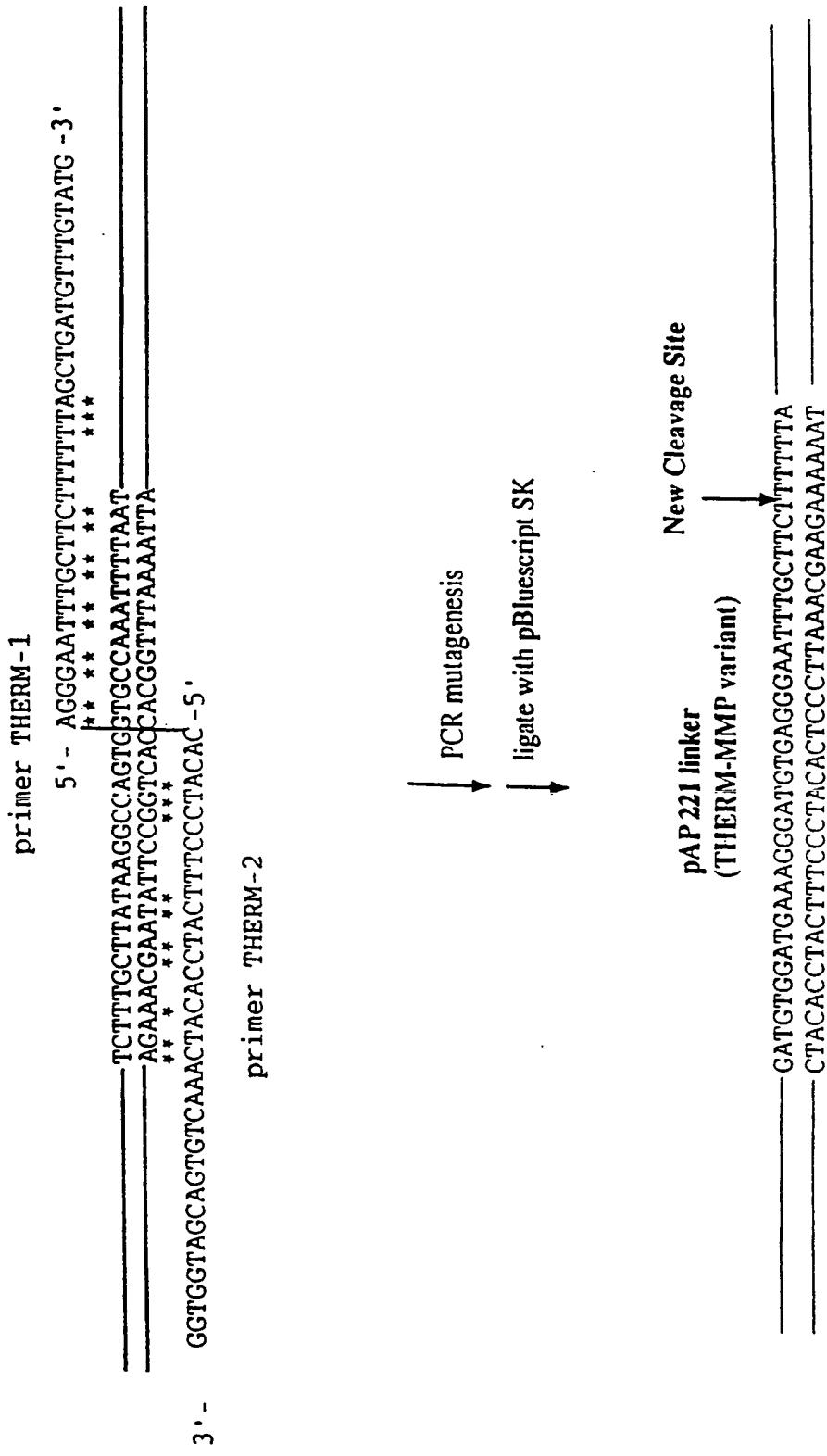
FIGURE 5D (CONT'D)

951 TGATGTTGTATGGATCCGTAGGCCATAGTCGTATCGTAGGTCAAATG
 ACTACAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACTACCTACCTCTAAGGTGTTGCCTTGCGTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCCGGTACGTTAGATTATGTCAGTTAGTCAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAATGAAAGTGTAACTACTTACG
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAAAACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATAACTAACGTATGACGACGT
 1201 ACTGATGCCACCCGTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCTTACCTGGTAGTATTAGG
 1251 CAGATCTAGTCTAGTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCTGTACCATGGTGTG
 1301 TTACAGTGCACCAACACATTATGCCGTTAGTCAGGGTGGCTTCAACT
 AATGTCACGTTGGTTGTAACAGGCAATCAGTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTTGGAAAACAATGTTGGTAACACCCGATAACAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTGTTATCACCTGTTACACCTATCTCTGACATCGTCACTTT
 1451 AGGCTGAACAAACAGTGGCTTTATGCAGATGGTCAATACGTCCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAAGGAGTC
 1501 CAAACCGAGATAATTGCCCTACAAGTGATTCTAATATAACGGAAACAGT
 GTTTGGCTCTATTAACGGATGTTCACTAAGATTATATGCCCTTGTCA
 1551 TGTAAAGATCCTCTTGTGGCCCTGCATCCTCTGGCAAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTTAAATTGTATAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTGGTAAACATATCACCTAACACAACTCTA
 1651 GTGAGGCGATGGATCCGAGCCTTAAACAAATCATTCTTACCCCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAAATATGGTACCAATTATTGTATAGACAGATTACT
 ACCACTGGGTTGGTTATACCAATGGTAATAAAACTATCTGTCTAATGA
 1751 CTCTTGCAGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAAA
 GAGAACGTCACACACAGGACGGTACTTTATCTACCGAATTATT
 1801 GGACATTGTAATTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTTAAACATTGACTTCCCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

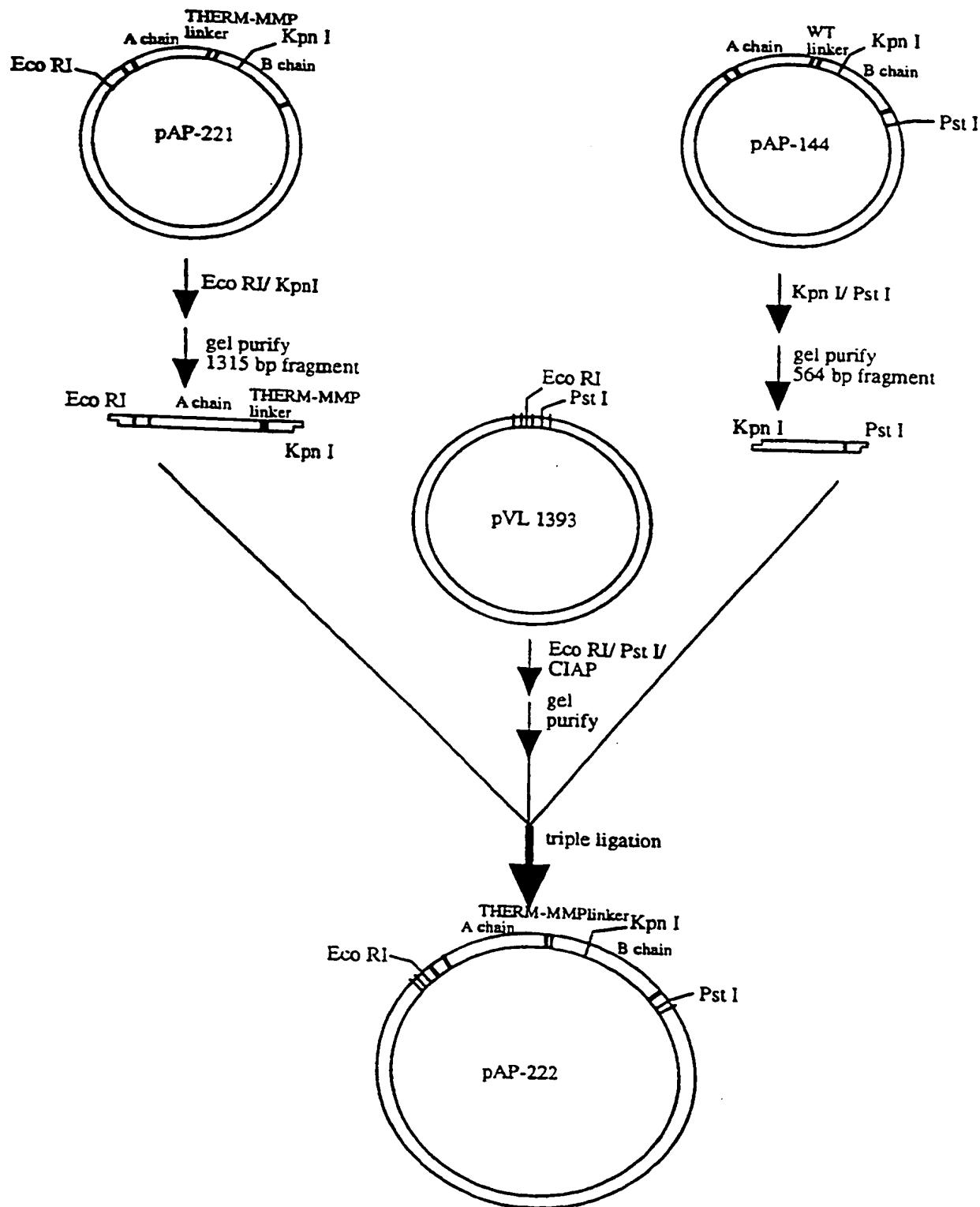
27/254

FIGURE 6A**SUBSTITUTE SHEET (RULE 26)**

28/254

FIGURE 6B**WT preprorcin linker****SUBSTITUTE SHEET (RULE 26)**

29/254

FIGURE 6C

SUBSTITUTE SHEET (RULE 26)

30/254

FIGURE 6D

10 20 30 40 50

```

1  GAATTCAATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTGGCCCTCCTTATGATAACATTACCTACATACGTCA

51  GGCAACATGGCTTGTGTTGGATCCACCTCAGGGTGGCTTTCACATTAG
   CGGTGTACCGAAACAAACCTAGTGGAGTCCCACCAGAAAGTGTAAATC

101 AGGATAACAACATATTCCCCAAACAAATACCCAATTATAAACCTTACCA
    TCCTATTGTTGTATAAGGGTTTGTATGGGTTAATATTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTCGCGG
    CGCCCCACGGTGACACGTTCGATGTGTTGAAATAGTCTCGACAAGCGCC

201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCACTGTGTTGCCAA
    AGCAAATTGTTGACCTCGACTACACTCTGACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACCTCTCA
    TGTCTCAACCAAACGGATATTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCAACCAATGCATA
    TTAGTACGTCTCGAAAGACAATGTAATCGCAGCCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGTGAAATAGCGCATATTCTTCATCCTGACA
    ACACCAAGCCGATGGCACGACCTTATCGGTATAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTCACTGATGTTCAAAAT
    TAGTCCTTCTACGTCTCGTTAGTGAAGTAGAAAAGTGAACACTAAGTTTA

451 CGATATACTCGCCTTGGTAATTATGATAGACTTGAACAACTTGC
    GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG
    ACCATTAGACTCTTTATAGCTCAACCCCTTACCAAGGTGATCTCCTCC

551 CTATCTCAGCGCTTATTATTACAGTACTGGTGGCACTCAGCTTCCA
    ACTGATAGTCGCGAAATAATAATGTATGACCAACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTCTTTATAATTGATCCAAATGATTTCAGAAGCAGCAAG
    GACCGAGCAAGGAAATATAACGTAGGTTACTAAAGTCTCGTCGTTTC

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
    TAAGGTTATATAACTCCCTTTACCGTGTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA
    CTAGACGTGGCTAGGATCGCATTATGTGAACCTTATCAACCCCTCT

751 CTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCA
    GAAAGGTGACGTTAAGTCTCAGATTGGTCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTCCAAATTCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
    ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTGATGTGGATGAAAGGGATGTGAGGGAAATTGCTTCTTT
    AGCAGTGTCAAACACTACACTTCCCTACACTCCCTAAACGAAGAAA

951 TTTAGCTGATGTTGTATGGATCCTGAGCCCAGTGCATCGTAGGTC

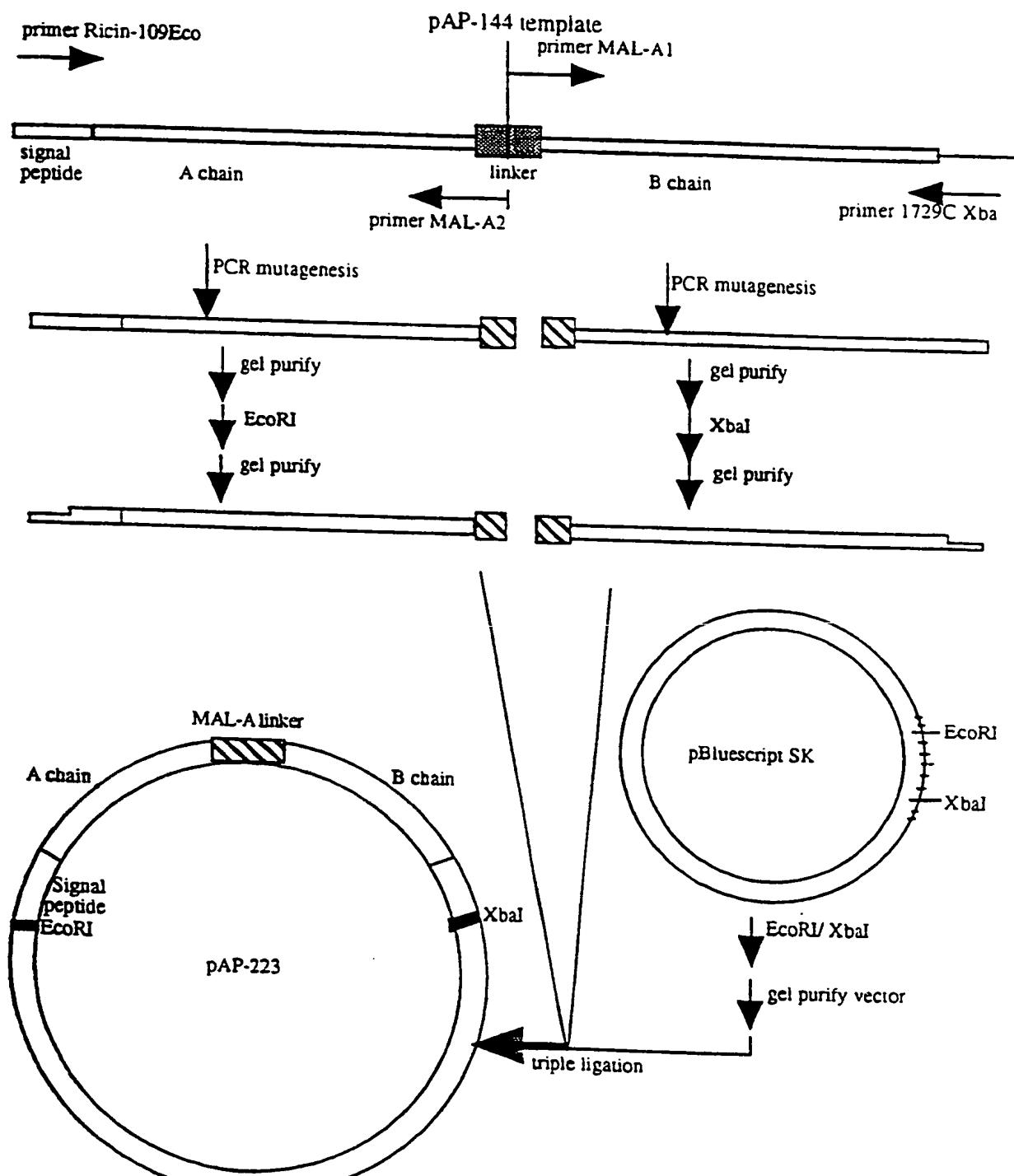
```

31/254

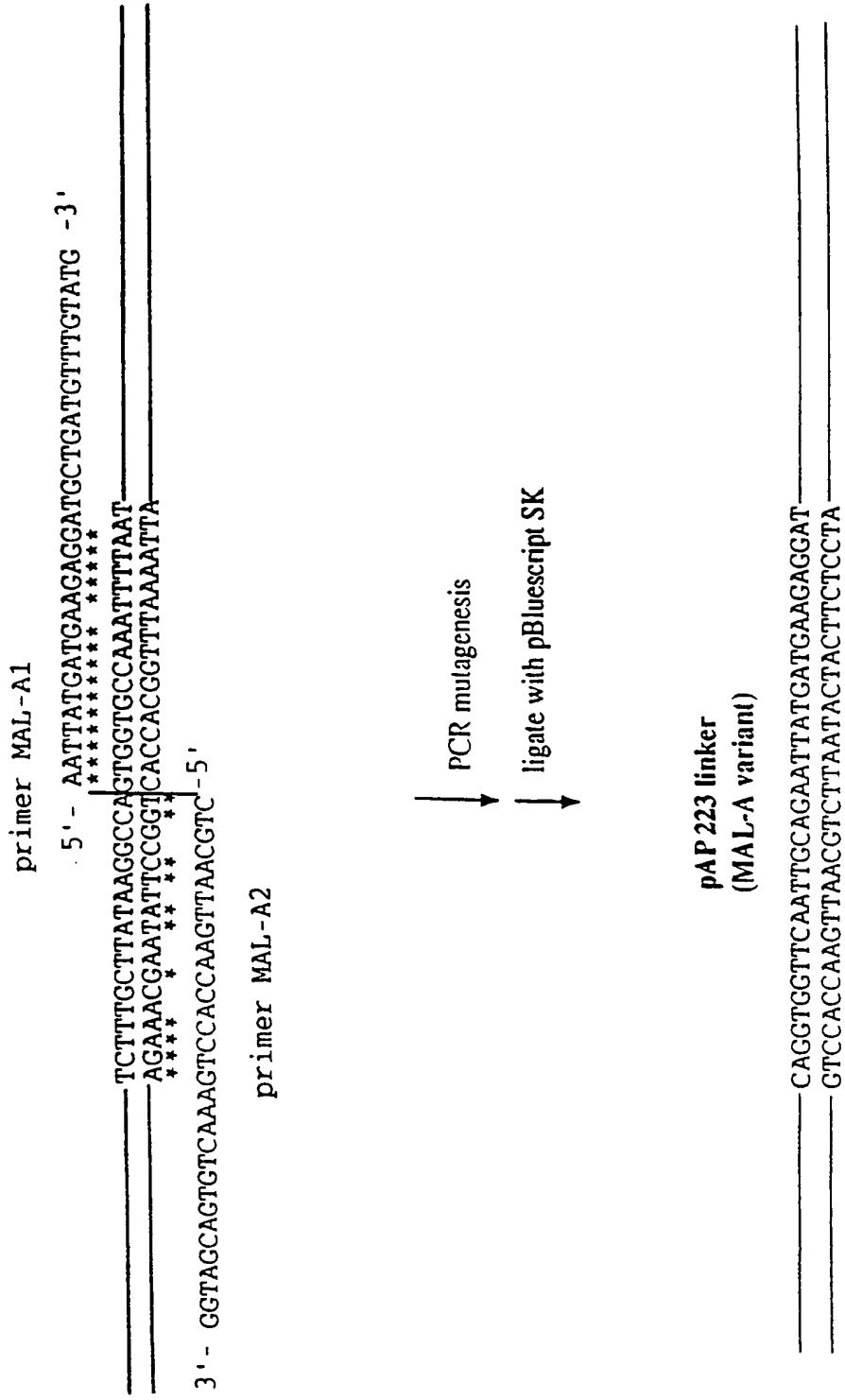
FIGURE 6D (CONT'D)

AAATCGACTACAAACATACTAGGACTCGGGTATCACGCATAGCATCCAG
 1001 GAAATGGTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAAC
 CTTTACCAAGATAACACAACATACAACTACATCCCTACCTTCAAGGTGTTGCCTTG
 1051 GCAATACAGTTGTCGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTG
 CGTTATGTCAACACCAGTACGTTCAAGATTATGTCTACGTTAGTCAGAC
 1101 GACTTTGAAAAGAGACAATACTATTGATCTAATGAAAGTGTAACTA
 CTGAAACTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGAT
 1151 CTTACGGGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACT
 GAATGCCCATGTCAGGCCCTCAGATAACACTACTAGATACTAACGTTATGA
 1201 GCTGCAACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCAT
 CGACGTTGACTACGGTGGCGACCGTTTACCCATTACCTTGGTAGTA
 1251 AAATCCCAGATCTAGTCTAGTTAGCAGCGACATCAGGGAACAGTGGTA
 TTTAGGGTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCAACCAT
 1301 CCACACTTACAGTGCAACCAACATTATGCCGTTAGTCAGGTTGGCTT
 GGTGTGAATGTCACGTTGGTTGTAACACGGCAATCAGTCCAACCGAA
 1351 CCTACTAATAACACAAACCTTTGTTACAACCATTGTTGGCTATATGG
 GGATGATTATTATGTGTTGGAAAACAATGTTGTAACAACCCGATATACC
 1401 TCTGTGTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCA
 AGACACGAACTCGTTTACCTGTTACCTACCTATCTCCTGACATCGT
 1451 GTGAAAAGGCTGAACAAACAGTGGGCTTTATGCAAGATGGTCAATACGT
 CACTTTCCGACTTGTGTCACCCGAGAAATACGTCACCAAGTTATGCA
 1501 CCTCAGCAAACCGAGATAATTGCTTACAAGTGTAACTACGGGA
 GGAGTCGTTGGCTCTATTAACGGAATGTTACCAAGATTATATGCCCT
 1551 AACAGTTGTTAAGATCCCTCTTGTGGCCCTGCATCCTGGCCAACGAT
 TTGTCAACAAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGTTGCTA
 1601 GGATGTTCAAGAATGATGGAACCATTTAAATTGTATAGTGGATTGGTG
 CCTACAAGTTCTACTACCTTGGTAAATTAAACATATCACCTAACAC
 1651 TTAGATGTGAGGCAGTCGGATCCGAGCCTAAACAAATCATTCTTACCC
 AATCTACACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGG
 1701 TCTCCATGGTGACCCAAACCAATATGGTTACCAATTATTTGATAGACAG
 AGAGGTACCACTGGTTGGTTTACCAATGTAATAAAACTATCTGTC
 1751 ATTACTCTCTGCACTGTGTTGTCCTGCCATGAAAATAGATGGCTTAAA
 TAATGAGAGAACGTCAACACACAGGACGGTACTTTTATCTACCGAATT
 1801 TAAAAAGGACATTGTAATTGTAACTGAAAGGACAGCAAGTTATATCG
 ATTTTCCTGTAACATTAAAACATTGACTTCCCTGTCGTTCAATATAGC
 1851 AATTCCCTGCAG
 TTAAGGACGTC

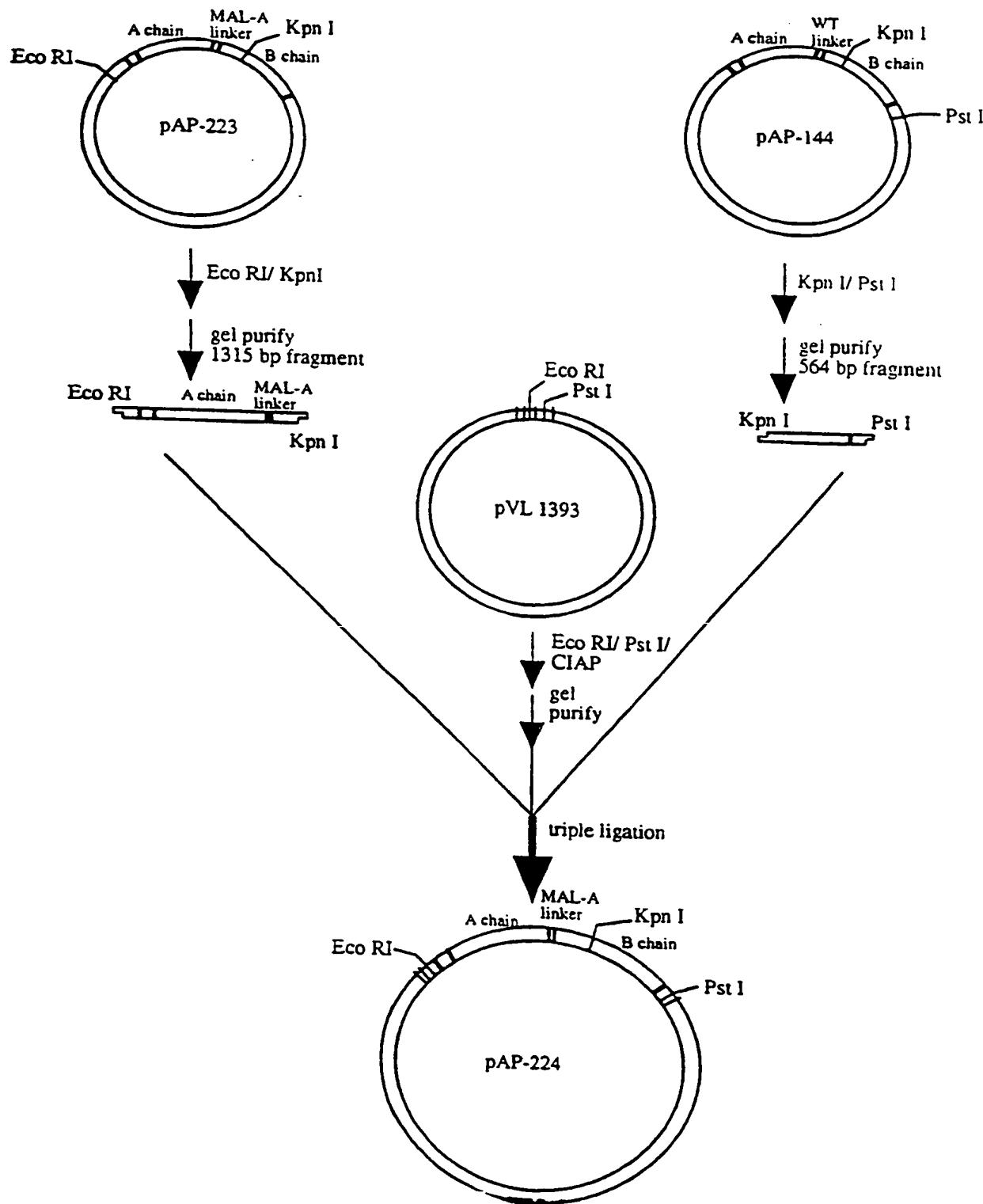
32/254

FIGURE 7A**SUBSTITUTE SHEET (RULE 26)**

33/254

FIGURE 7B**WT preprorocin linker****SUBSTITUTE SHEET (RULE 26)**

34/254

FIGURE 7C

SUBSTITUTE SHEET (RULE 26)

35/254

FIGURE 7D

1	10	20	30	40	50
---	----	----	----	----	----

```

GAATTCATGAAACCGGGAGGAATACTATTGTAAATATGGATGTATGCAGT
CTTAAGTACTTTGCCCTCCTTATGATAACATTATACTACATACGTCA

51 GGCAACATGGCTTGTGGATCCACCTCAGGGTGGTCTTCACATTAG
CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC

101 AGGATAACAAACATATTCCCCAAACAATACCAATTATAAACTTTACACAC
TCCTATTGTTGTATAAGGGTTTGTATGGTTAATATTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTGCCGG
CGCCCACGGTGACACGTTCGATGTGTTGAAATAGTCTCGACAAGCGCC

201 TCGTTAACAAACTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGTTGCCTATAAAACCAACGGTTTATTTAGTTGAACCTCTCA
TGTCTCAACCAAACGGATATTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAAATAGCGCATATTCTTCATCCTGACA
ACACCAAGCCGATGGCACGACCTTATCGGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCGAAGCAATCACTCATCTTCACTGATGTTCAAAT
TAGTCCTTCTACGTCTCGTAGTGGAGTAGAAAGTGAACACTAACAGTTTA

451 CGATATAACATTGCCCTTGGGTAATTATGATAGACTTGAACAACTTGC
GCTATATGTAAGCGAACCAACCATTAATACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGAAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTTTATAGCTCAACCCCTTACCAAGGTGATCTCCTCC

551 CTATCTCAGCGCTTATTACAGTACTGGTGGCACTCAGCTTCAACT
GATAGAGTCGCGAAATAATAATGTATGACCAACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTCCCTTATAATTGACATCCAAATGATTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAACGTAGGTTACTAAAGTCTCGTGTTC

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGGTATATAACTCCCTTTACGCGTGTCTTAATCCATGTTGGCCT

701 GATCTGCACCAAGATCCTAGCGTAATTACACTTGAAGAATAGTTGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTCCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTCCAAATTCACTGAGTGTACGATGTGAGTA
AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTCAAGGTGGTCAATTGAGAATTATGATGAAGAGGATGC
AGCAGTGTCAAAGTCCACCAAGTTAACGTCTTAATACTACTTCTCCTACG

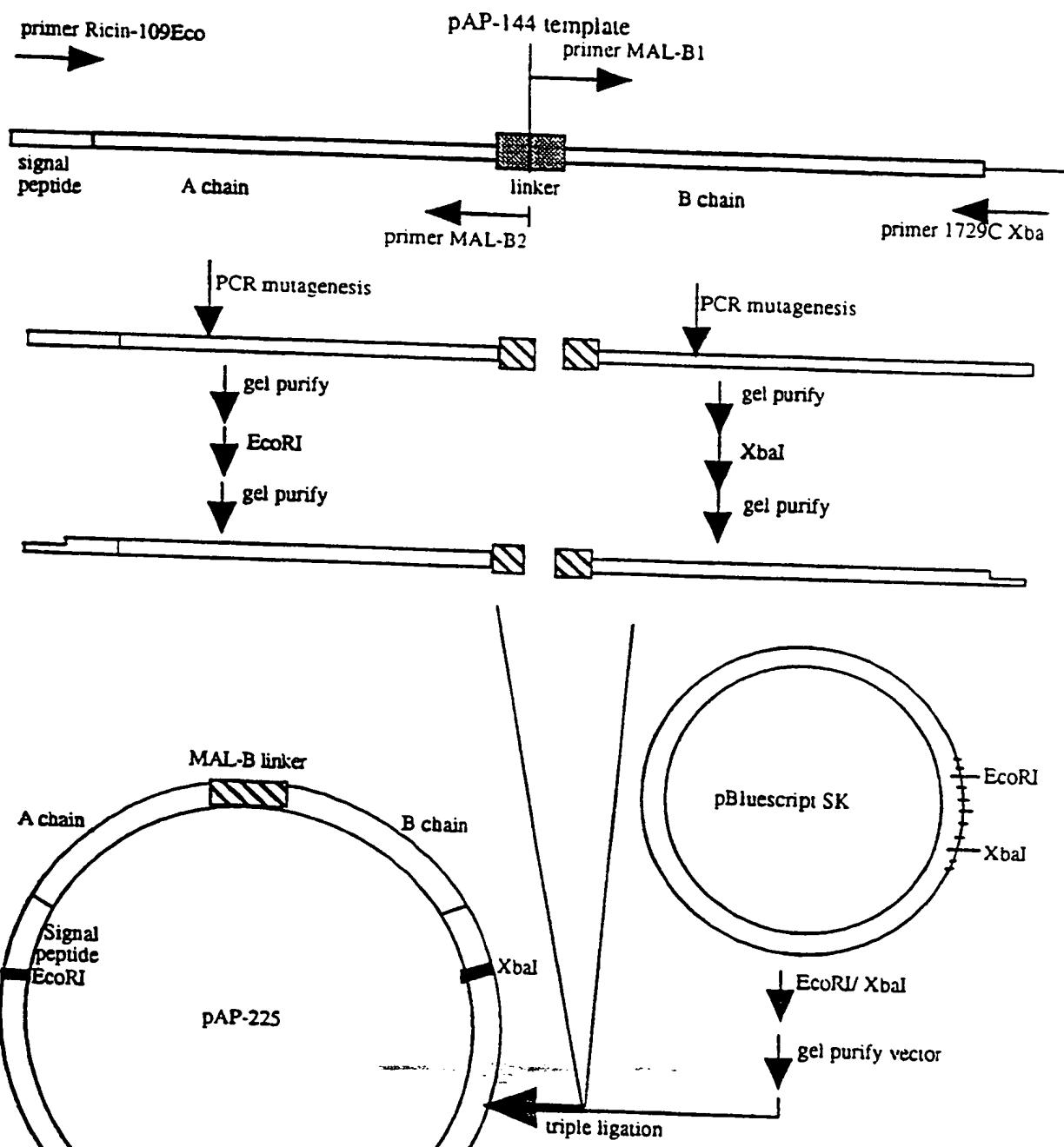
```

36/254

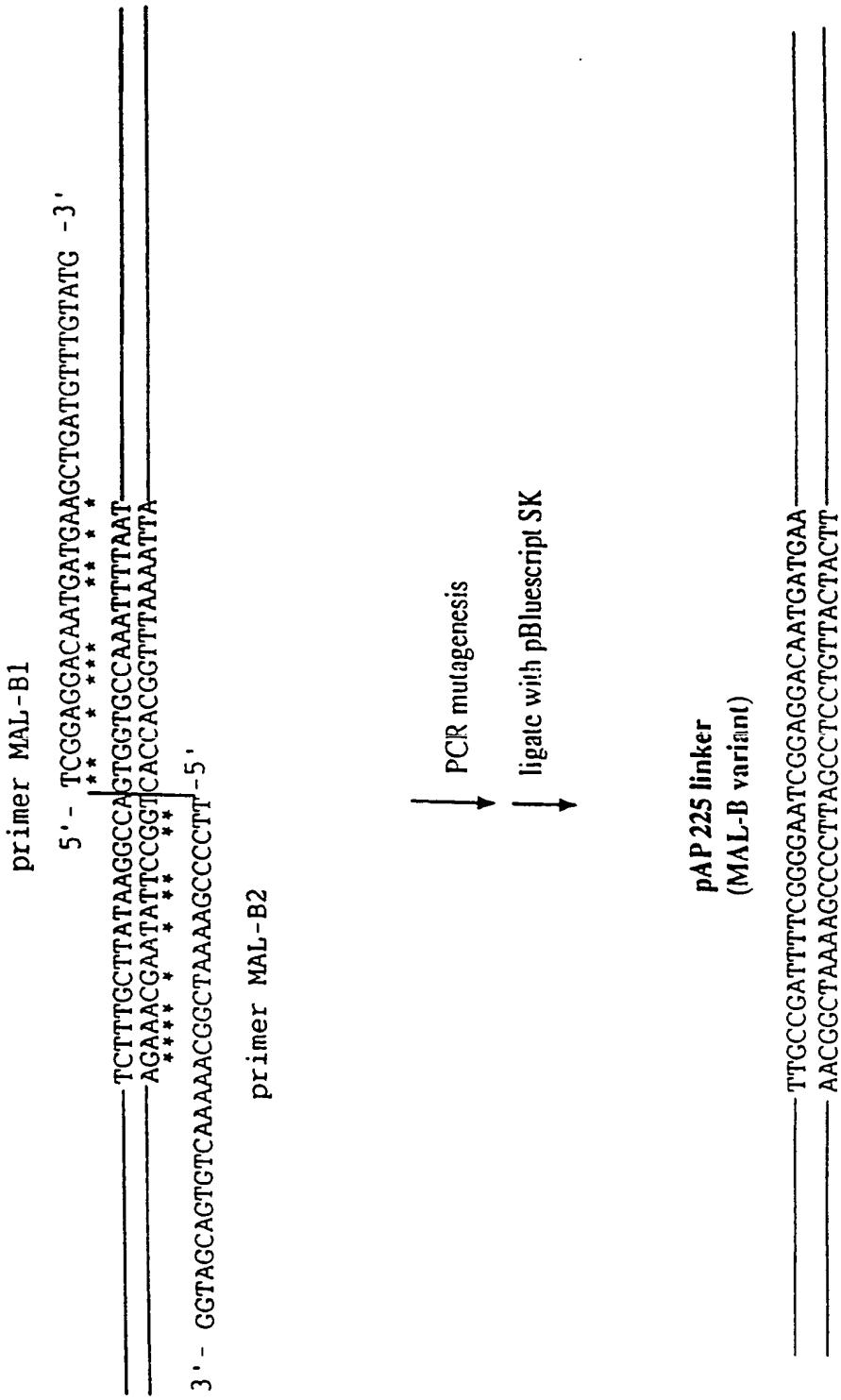
FIGURE 7D (CONT'D)

951 TGATGTTGTATGGATCCTGAGCCCATACTGCGTATCGTAGGTGAAATG
 ACTACAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACTACATCCCTACCTCTAAGGTGTTGCCTTGCGTTAT
 1051 CAGTTGTGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTAGATTATGTCAGTTAGTCAGACTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAATGAAAGTGTAACTACTAACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCACATCATAAATCC
 TGACTACGGTGGCGACCGTTTACCCATTACCTTGGTAGTATTAGG
 1251 CAGATCTAGTCTAGTTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTGTCACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTATGCCGTTAGTCAAGGTTGGCTTCTACT
 AATGTCACGTTGGTTGTAACACGGCAATCAGTTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTGTTACAACCAATTGTTGGCTATATGGTCTGTG
 TTATTATGTTGAAAACAATGTTGGTAACACCCGATATACCAAGACAC
 1401 CTTGCAAGCAAATAGGGACAAGTATGGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTCTTATCACCTGTTACACCTATCTCCTGACATCGTCACTTT
 1451 AGGCTGAACAAACAGTGGCTCTTATGCAGATGGTCAATACGTCCTCAG
 TCCGACTTGTGTCACCCGAGAAACAGTCTACCAAGTTATGCAAGGAGTC
 1501 CAAAACCGAGATAATTGCCCTACAAGTGATTCTAATATACGGGAAACAGT
 GTTTGGCTCTATTACCGGAATGTTACAGATTATATGCCCTTGTCA
 1551 TGTTAAGATCCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCAATTAAATTGTTACAGGATTGGGTAGAT
 AGTTCTTACCTGGTAAACATACGTTACACCTAACCAATCTA
 1651 GTGAGGCAGTCGGATCCGAGCCTAAACAAATCATTCTTACCCCTCTCCA
 CACTCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAAATGGTACCAATTGGTATAGACAGATTACT
 ACCACTGGGTTGGTTACCAATGTTAAACATATCTGCTAATGA
 1751 CTCTTGAGTGTGTCCTGCCATGAAAATAGATGGCTAAATAAAAAA
 GAGAACGTACACACACAGGACGGTACCTTATCTACCGAATTATTTT
 1801 GGACATTGTAACATTGTAACGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAACATTGACTTCTGCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

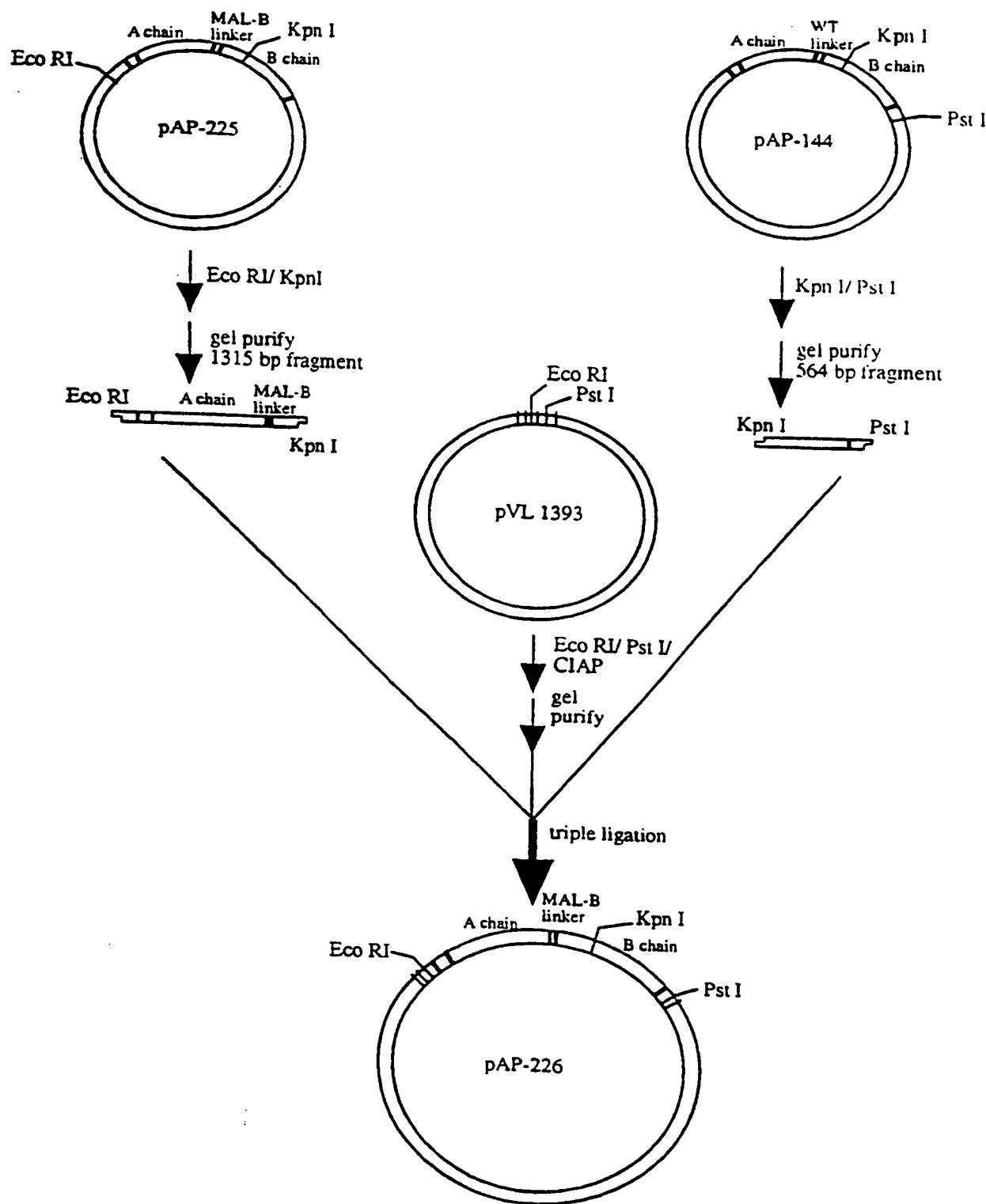
37/254

FIGURE 8A**SUBSTITUTE SHEET (RULE 26)**

38/254

FIGURE 8B**WT preprorocin linker****SUBSTITUTE SHEET (RULE 26)**

39/254

FIGURE 8C

SUBSTITUTE SHEET (RULE 26)

40/254

FIGURE 8D

10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT			
	CTTAAGTACTTGGCCCTCCTTATGATAACATTATACCTACATACGTCA			
51	GGCAACATGGCTTGTGATCCACCTCAGGGTGGCTTCACATTAG			
	CCGTTGTACCGAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTATC			
101	AGGATAACAAACATATTCCCCAAACAATACCCAATTATAAACCTTACACAA			
	TCCTATTGTTGTATAGGGGTTGTTATGGGTTAATATTGAAATGGTGT			
151	GCGGGTGCCACTGTCAAAGCTACACAAACTTATCAGAGCTGTTCGCGG			
	CGCCCACGGTGACACGTTGATGTGTTGAAATAGTCTCGACAAGCGCC			
201	TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAAGTGTGCAA			
	AGCAAATTGTTGACCTCGACTACACTCTGACTATATGGTCACAACGGTT			
251	ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACTCTCA			
	TGTCTCAACCAAACGGATATTGGTTGCCAAATAAACTCAACTTGAGAGT			
301	AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA			
	TTAGTACGTCTCGAAAGACAATGTAATCGCACCTACAGTGGTTACGTAT			
351	TGTGGTCGGCTACCGTGTGGAAATAGCGCATATTCTTACATCCTGACA			
	ACACCAGCCGATGGCACGACCTTATCGGTATAAAGAAAGTAGGACTGT			
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTCACTGATGTTCAAAAT			
	TAGTCCTTCTACGTCTCGTTAGTGAGTAGAAAGTGAACATCAAGTTTA			
451	CGATATACATTGCCCTTGGGGTAATTATGATAGACTGAAACAACCTGC			
	GCTATATGTAAGCGGAAACCACCAATTAAACTATCTGAACCTGTTGAACG			
501	TGGTAATCTGAGAGAAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG			
	ACCATTAGACTCTCTTTATAGCTAACCCCTTACCGGTGATCTCCCTCC			
551	CTATCTCAGCGCTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC			
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA			
601	CTGGCTCGTCTTATAATTGATCCAAATGATTCAGAAGCAGCAAG			
	GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTCGTT			
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA			
	TAAGGTTATATAACTCCCTCTTACCGGTGCTCTAATCCATGTTGGCCT			
701	GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAAATAGTTGGGGAGA			
	CTAGACGTGGTCTAGGATCGCATTAAATGTAACCTTATCAACCCCTCT			
751	CTTTCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGTCTAGTCCAAT			
	GAAAGGTGACGTTAAGTCTCAGATTGTTCTCGGAAACGATCAGGTTA			
801	TCAACTGCAAAGACGTAATGGTTCAAATTCAAGTGTGACGATGTGAGTA			
	AGTTGACGTTCTGATTACCAAGGTTAAGTCACACATGCTACACTCAT			
851	TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA			
	ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACCGTGAGGTGGT			
901	TCGTCACAGTTTGCCTGATTTGGGGAAATCGGAGGACAATGATGAAGC			
	AGCAGTGTCAAAACGGCTAAAAGCCCTTAGCCTCCTGTTACTACTTCG			

41/254

FIGURE 8D (CONT'D)

951 TGATGTTGTATGGATCCTGAGCCCATACTGCGTATCGTAGGTCGAATG
 ACTACAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC

 1001 GTCTATGTGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACAAATCCCTACCTCTAAGGTGTTGCCTTGCCTTAT

 1051 CAGTTGTGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTCAAGATTATGTCTACGTTAGTCGAGACCTGAAA

 1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC

 1151 GGTACAGTCGGGAGTCTATGTGATGATCTATGATTGCAAACTGCTGCA
 CCATGTCAGGCCCTCAGATAACACTAGATAACTAACGTTATGACGACGT

 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGCGACCCTTATACCCCTATTACCTTGGTAGTATTAGG

 1251 CAGATCTAGTCTAGTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTGTCACCATGGTGTG

 1301 TTACAGTGCAAACCAACATTATGCCCTTAGTCAGGTTGGCTTCAACT
 AATGTCACGTTGGTTGTAATACGGAATCAGTCCAACCGAAGGATGA

 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAACAAATGTTGTAACAAACCCGATATACCAGACAC

 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAA
 GAACGTTCGTTATCACCTGTTACACCTATCTCCTGACATCGTCACTTT

 1451 AGGCTGAACACAGTGGCCTTTATGCAGATGGTCATAACGTCCCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAAGGAGTC

 1501 CAAACCGAGATAATTGCCCTACAAGTGATTCTAATATACGGAAACAGT
 GTTTGGCTCTATTAAAGGAATGTTCACTAAGATTATATGCCCTTGTCA

 1551 TGTTAAGATCCTCTTGTGGCCCTGCATCCTCTGGCAAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

 1601 TCAAGAATGATGGAACCATTTAAATTGTATAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTGGTAAATTTAAACATATCACCTAACCAACAACTCA

 1651 GTGAGGCATGGATCCGAGCCTTAAACAAATCATTCTTACCCCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT

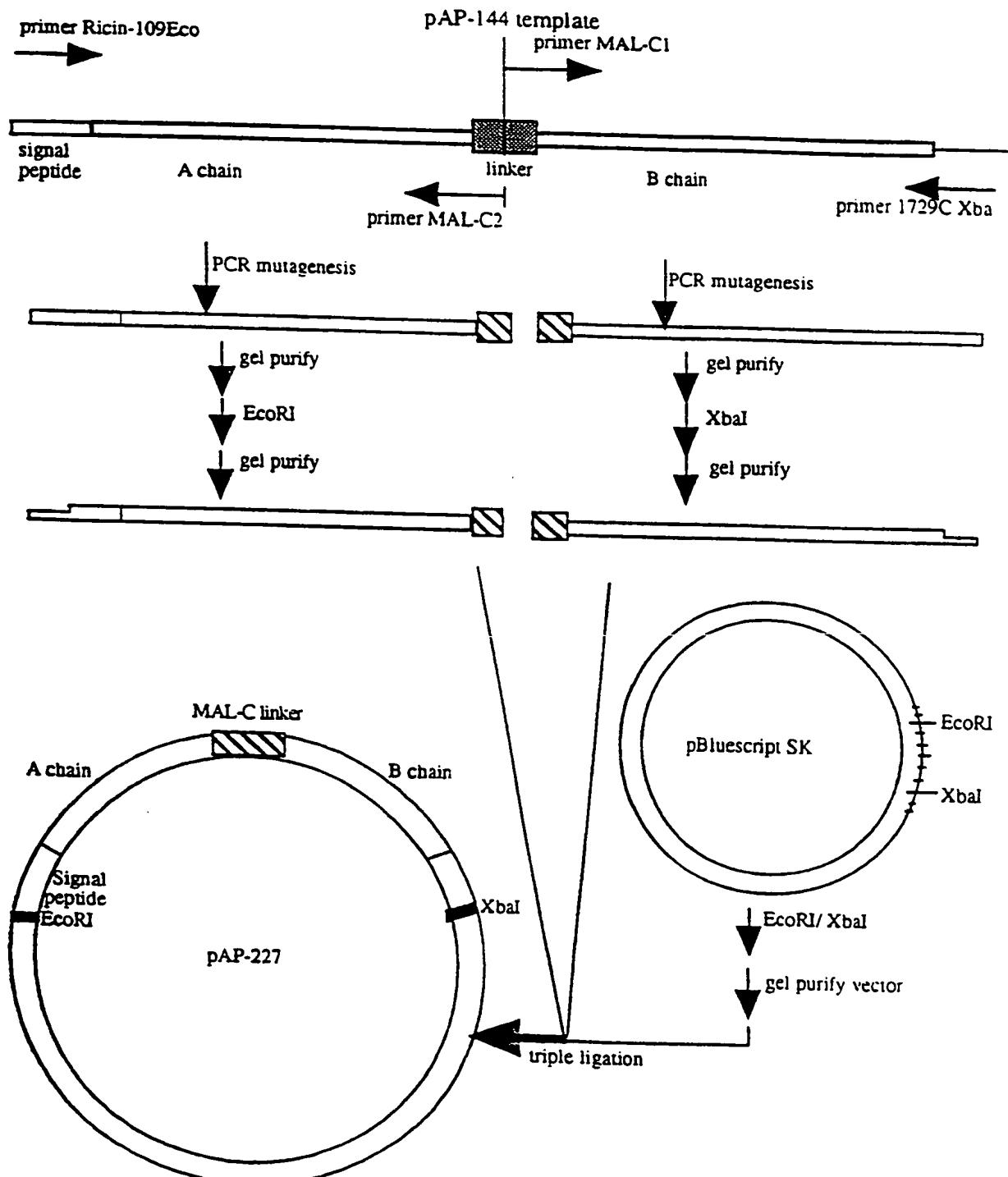
 1701 TGGTGACCCAAACAAATATGGTACCAATTATTTGATAGACAGATTACT
 ACCACTGGGTTGGTTATACCAATGGTAATAAAACTATCTGTCTAATGA

 1751 CTCTTGCAGTGTGTCCTGCCATGAAAATAGATGGCTAAATAAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTT

 1801 GGACATTGTAATTTGTAACGTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAACATTGACTTCCCTGTCGTTCAATATAGCTTAAGG

 1851 TGCAG
 ACGTC

42/254

FIGURE 9A**SUBSTITUTE SHEET (RULE 26)**

43/254

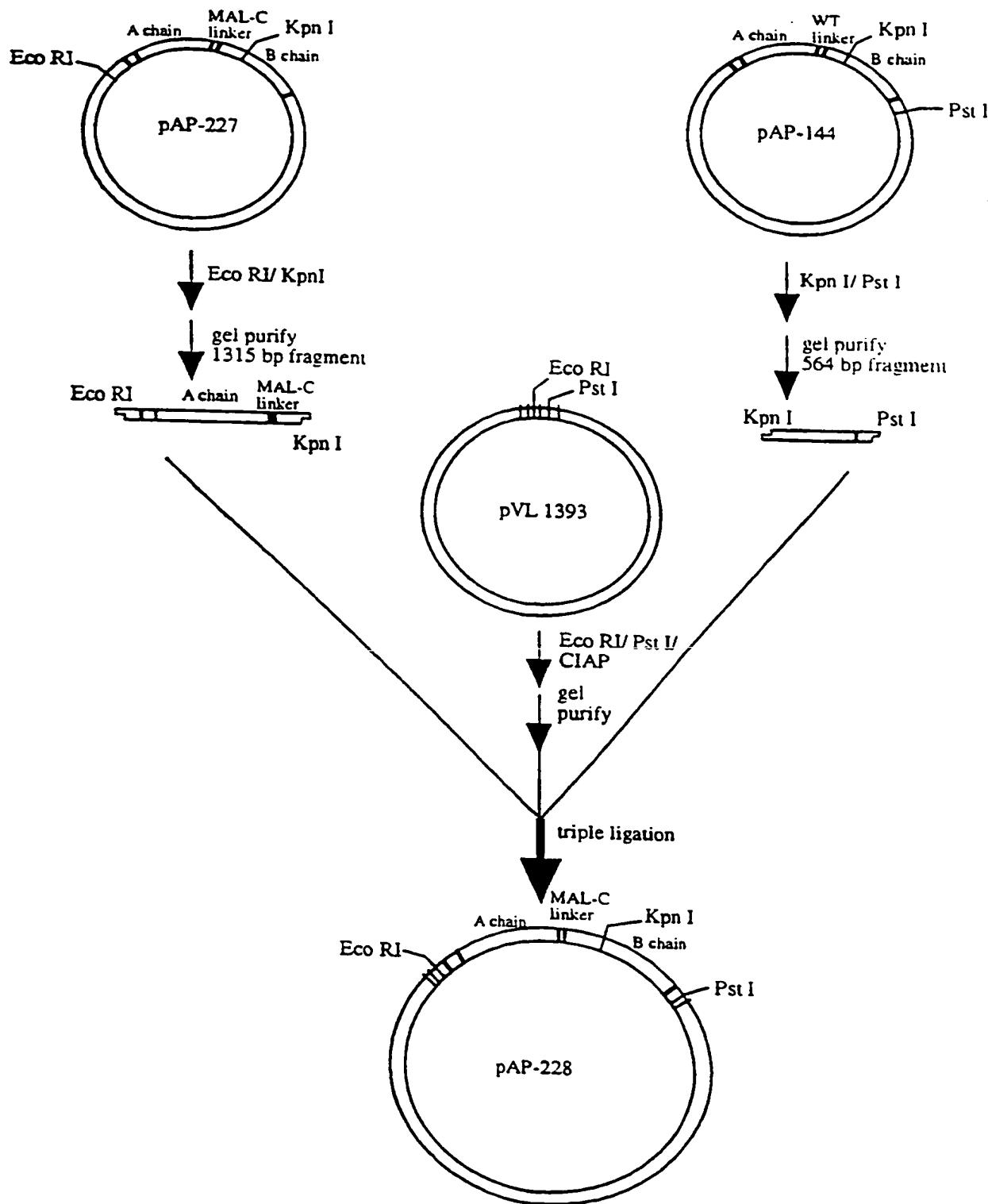
FIGURE 9B

WT prorocin linker



SUBSTITUTE SHEET (RULE 26)

44/254

FIGURE 9C

SUBSTITUTE SHEET (RULE 26)

45/254

FIGURE 9D

10	20	30	40	50
1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
CTTAAGTACTTTGCCCTCCTTATGATAACATTATACTACACATACGTCA				
51 GGCAACATGGCTTGTGATCCACCTCAGGGTGGCTTTCACATTAG				
CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAAGAAAGTGTAA				
101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACCTTACCA				
TCCTATTGTTGTATAAGGGGTTGTTATGGGTTAATATTGAAATGGTGT				
151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTCGCG				
CGCCCCACGGTGACACGTTCGATGTGTTGAAATAGTCTCGACAAGCGCC				
201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATAACCAGTGTGCCAA				
AGCAAATTGTTGACCTCGACTACACTGTACTATATGGTCACAACGGTT				
251 ACAGAGTTGGTTGCCCTATAAACCAACGGTTATTTAGTTGAACCTCTCA				
TGTCTCAACCAACGGATATTGGTTGCCAAATAAACTCAACTTGAGAGT				
301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351 TGTGGTCGGTACCGTGTGGAAATAGCGCATATTTCTTCATCCTGACA				
ACACCAGCCGATGGCACGACCTTATCGGTATAAAGAAAGTAGGACTGT				
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTCACTGATGTTAAAAT				
TAGTCCTTCTACGTCTCGTTAGTAGAAAAGTGAACACTACAAGTTTA				
451 CGATATACATTGCCCTTGGTGGTAATTATGATAGACTTGAACAACTTGC				
GCTATATGTAAGCGGAAACCAACCATTAATACTATCTGAACCTGTTGAACG				
501 TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG				
ACCATTAGACTCTTTATAGCTAACCCCTTACAGGTGATCTCCCTCC				
551 CTATCTCAGCGCTTTATTACAGTACTGGTGGCACTCAGCTTCAA				
GATAGAGTCGCAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601 CTGGCTCGTCTTATAATTGATCCAAATGATTCAGAAGCAGCAAG				
GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTCTTC				
651 ATTCAAATATGGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
TAAGGTTATATAACTCCCTTTACCGTGCTTTAATCCATGTTGGCCT				
701 GATCTGCACCAAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA				
CTAGACGTGGCTAGGATCGCATTATGTGAACCTTATCAACCCCTCT				
751 CTTTCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGTCTAGTCAA				
GAAAGGTGACGTTAAGTTCTCAGATTGGTCTCGGAAACGATCAGGTTA				
801 TCAACTGCAAAGACGTAATGGTCCAAATTCAAGTGTGACGATGTGAGTA				
AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT				
851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA				
ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACCGTGGAGGTGGT				
901 TCGTCACAGTTCAAGGTGGTTACAGGGGAAGCGATATCAGTTACTATGGC				
AGCAGTGTCAAAGTCCACCAATGTCCTCGCTATAGTCAATGATAACCG				

46/254

FIGURE 9D (CONT'D)

951 TGATGTTGTATGGATCCTGAGCCCATACTGCGTATCGTAGGTCGAAATG
 ACTACAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC

 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACTACCTACCTCTAAGGTGTTAGTCGAGACCTGAAA

 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTAGATTATGTCAGTTAGTCGAGACCTGAAA

 1101 GAAAAGAGACAATACTATTGATCTAAATGGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC

 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATAACACTACTAGATAACTAACGTTATGACGACGT

 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCTTACCTGGTAGTATTAGG

 1251 CAGATCTAGTCTAGTTTAGCAGCGACATCAGGGAACAGTGGTACCCACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCTTGTCACCATGGTGTG

 1301 TTACAGTCAAACCAACATTATGCCCTAGTCAGGTTGGCTTCCTACT
 AATGTCACGTTGTTGTAATAACGGCAATCAGTCCAACCGAAGGATGA

 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTTGGAAAACAATGTTGTAACAACCGATATACCAGACAC

 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTAAAA
 GAACGTTGTTATCACCTGTTACACCTATCTCCTGACATCGTCACCTT

 1451 AGGCTGAAACAACAGTGGGCTTTATGCAGATGGTTCAATACTCCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

 1501 CAAAACCGAGATAATTGCCCTACAAGTGTAACTATACGGGAAACAGT
 GTTTGGCTCTATAACGGAATGTTCAACTAAGATTATATGCCCTTGTC

 1551 TGTTAAGATCCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTTAGGAGACCGGTTGCTACCTACA

 1601 TCAAGAATGATGGAACCATTAAATTGTATAGTGGATTGGTGTAGAT
 AGTTCTTAACCTTGGTAAATTAAACATATCACCTAACCAACAACTTA

 1651 GTGAGGCATGGATCCGAGCCTAAACAAATCATTCTTACCCCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT

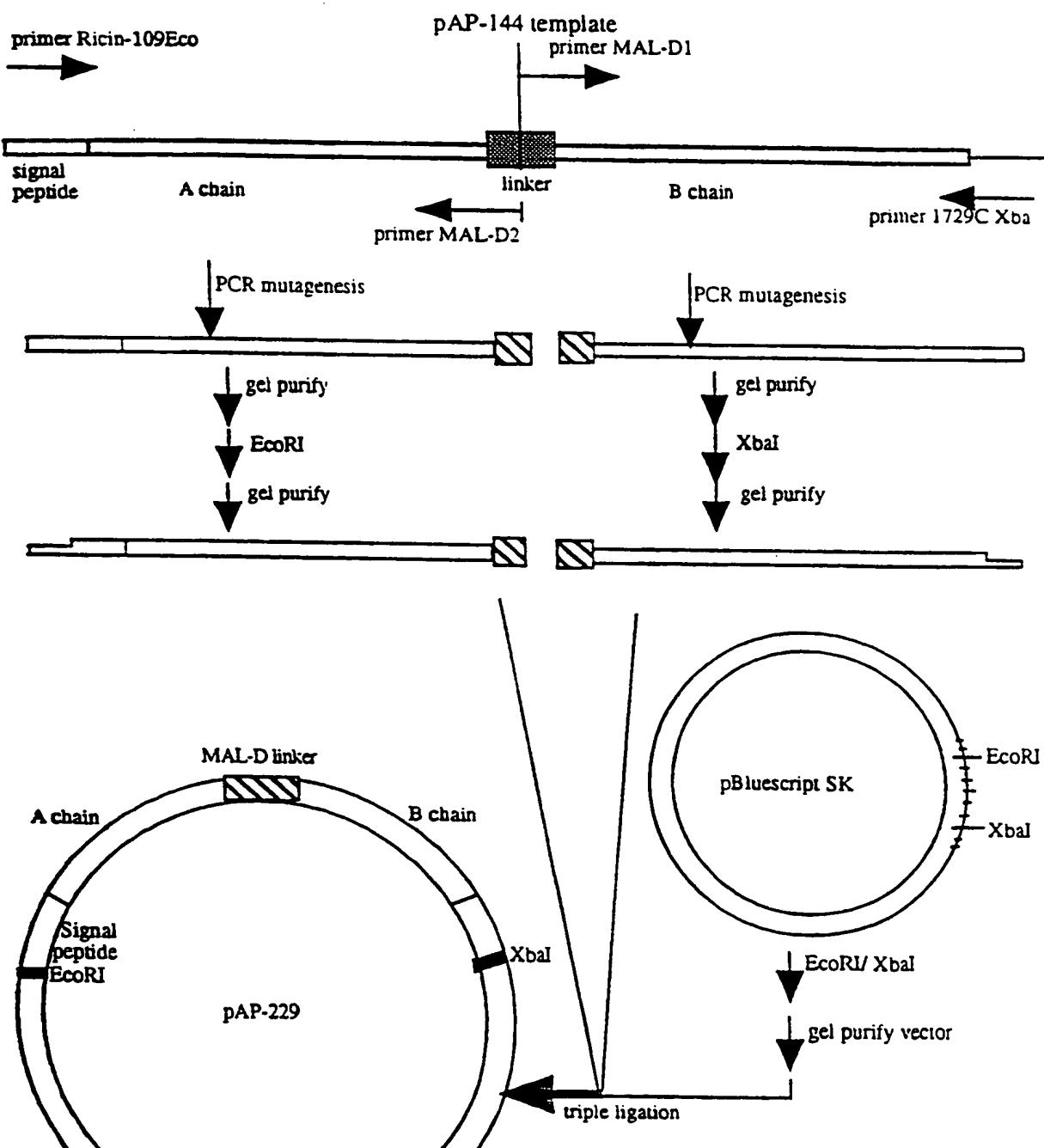
 1701 TGGTGACCCAAACCAAATATGGTTACCATTTGATAGACAGATTACT
 ACCACTGGGTTGGTTATACCAATGTAATAAAACTATCTGTCTAATGA

 1751 CTCTTGCAGTGTGTGTCTGCCATGAAAATAGATGGCTAAATAAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTT

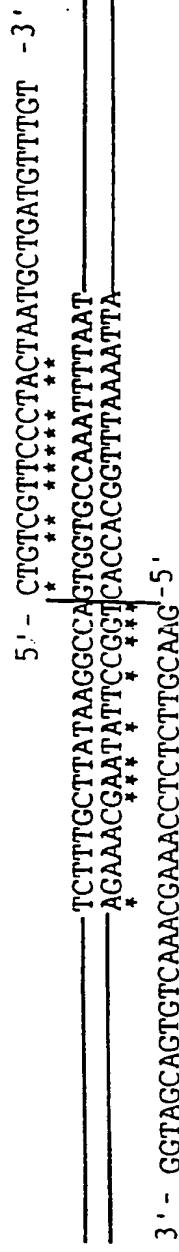
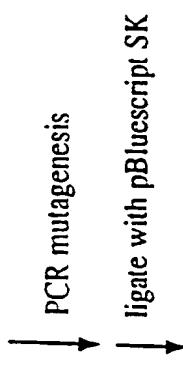
 1801 GGACATTGTAATTGGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAAACATTGACTTCTGTCGTTCAATATAGCTTAAGG

 1851 TGCAG
 ACGTC

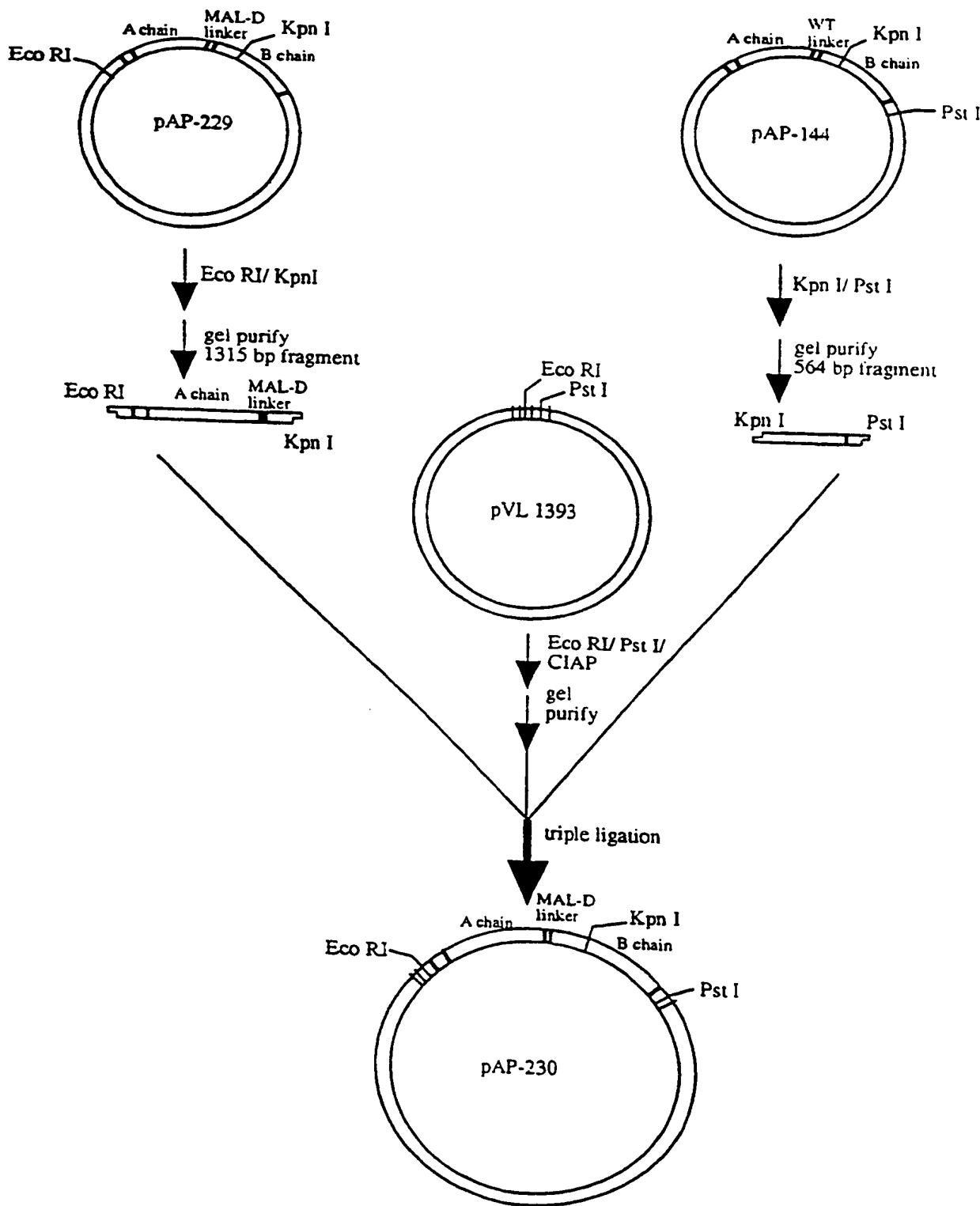
47/254

FIGURE 10A**SUBSTITUTE SHEET (RULE 26)**

48/254

FIGURE 10B**WT preprorcin linker****primer MAL-D1****primer MAL-D2****pAP 229 linker
(MAL-D variant)**

49/254

FIGURE 10C

SUBSTITUTE SHEET (RULE 26)

50/254

FIGURE 10D

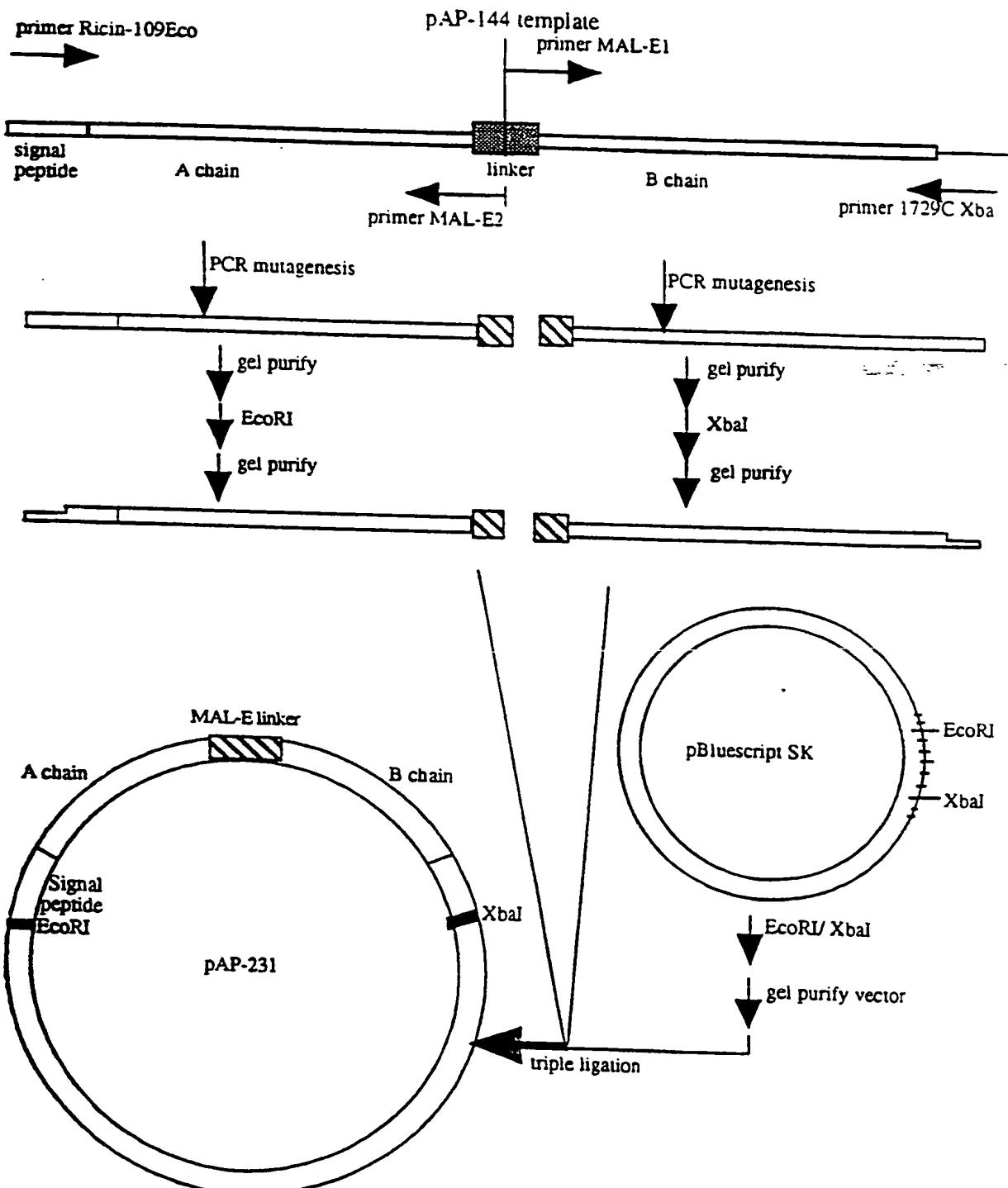
10	20	30	40	50
1 GAATT	CTGAAACCGGGAGGAATACTATTGTAATATGGATGTATGCAGT			
	CTTAAGTACTTGGCCCTCTTATGATAACATTATACCTACATACGTCA			
51 GGCAACATGGCTTGTTGGATCCACCTCAGGGTGGCTTTCACATTAG				
CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAA				
101 AGGATAACAAACATATTCCCCAAACAATACCCAATTATAAATTTACCACA				
TCCTATTGTTGTATAAGGGTTTGTATGGTTAATATTGAAATGGTGT				
151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTCGCG				
CGCCCACGGTGACACGTTCGATGTGTTGAAATAGTCTCGACAAGCGCC				
201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAAGTGTGCCAA				
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT				
251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACTCTCA				
TGTCTCAACCAAACGGATATTGGTTGCCAAATAAAATCAACTTGAGAGT				
301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
TTAGTACGTCTCGAAAGACAATGTAATCGCACCTACAGTGGTTACGTAT				
351 TGTGGTCGGCTACCGTGTGGAAATAGCGCATATTCTTCATCCTGACA				
ACACCAAGCCGATGGCACGACCTTATCGCGTATAAAGAAAGTAGGACTGT				
401 ATCAGGAAGATGCAAGCAATCACTCATCTTCACTGATGTTAAAAT				
TAGTCCTCTACGTCTCGTTAGTGAGTAGAAAGTGAACACTAAGTTTA				
451 CGATATACTTCGCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC				
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG				
501 TGGTAATCTGAGAGAAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG				
ACCATTAGACTCTCTTATAGCTCAACCCTTACCGGTGATCTCCTCC				
551 CTATCTCAGCGCTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601 CTGGCTCGTCCTTATAATTGATCCAAATGATTTAGAAGCAGCAAG				
GACCGAGCAAGGAAATATAAACGTAGGTTACTAAAGTCTCGTCGTTC				
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
TAAGGTTATATAACTCCCTTTACCGCGTCTTAATCCATGTTGGCCT				
701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA				
CTAGACGTGGCTAGGATCGCATTATGTGAACCTTATCAACCCCTCT				
751 CTTTCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT				
GAAAGGTGACGTTAAGTTCTCAGATTGGTCTCGGAAACGATCAGGTTA				
801 TCAACTGCAAAGACGTAATTGGTCCAAATTCACTGTTACGATGTGAGTA				
AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT				
851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA				
ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACGCGTGGAGGTGGT				
901 TCGTCACAGTTGCTTGAGAGAACTGTTCTGTCGTTCCCTACTAATGC				
AGCAGTGTCAAACGAAACCTCTCTTGCAGGACAGCAAGGGATGATTACG				

51/254

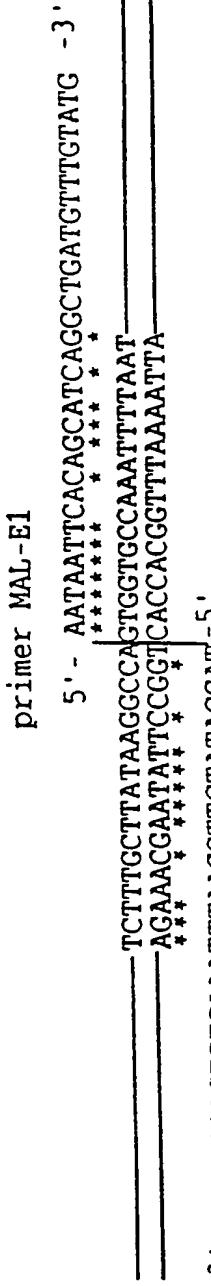
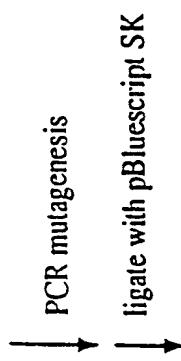
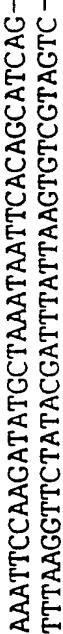
FIGURE 10D (CONT'D)

951 TGATGTTGTATGGATCCTGAGCCCATACTGCGTATCGTAGGTCGAAATG
ACTACAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC
1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACATACTACATCCCTACCTCTAAGGTGTTGCCTTGCGTTAT
1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTAGATTATGTCAGTCTACGTTAGTCGAGACCTGAAA
1101 GAAAAGAGACAATACTATTGATCTAAATGGAAAGTGTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTAGATACTAACGTTATGACGACGT
1201 ACTGATGCCACCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGCGACCGTTATACCTTACCTTGGTAGTATTAGG
1251 CAGATCTAGTCTAGTTTAGCAGCGACATCAGGGAACAGTGGTACCCACAC
GTCTAGATCAGATCAAATCGTCGCTGAGTCCCTGTCACCATGGTGTG
1301 TTACAGTGCAAACCAACATTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTGGTTGTAATAACGGCAATCAGTCCAACCGAAGGATGA
1351 AATAATACACAAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
TTATTATGTGTTGAAAACATGTTGTAACAACCCGATATAACCAGACAC
1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGA
AAACGTTCGTTATCACCTGTTACACCTATCTCCTGACATCGTCACTT
1451 AGGCTGAACAAACAGTGGCTTTATGCAGATGGTCAATACTGCCTCAG
TCCGACTTGTGTCACCCGAGAAAATACGTCTACCAAGTTATGCAGGAGTC
1501 CAAAACCGAGATAATTGCCCTACAAGTGATTCTAATATACGGGAAACAGT
GTTTGCGCTTAAACGGAAATGTCACTAAGATTATATGCCCTTGTC
1551 TGTTAAGATCCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
1601 TCAAGAATGATGGAACCATTAAATTGTATACTGGATTGGTGTAGAT
AGTTCTTACTACCTTGGTAAATTAACATATCACCTAACCAACAACTCTA
1651 GTGAGGGGATCGGATCCGAGCCTTAAACAAATCATTCTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT
1701 TGGTGACCCAAACCAAATATGGTACCAATTATTTGATAGACAGATTACT
ACCACTGGTTGGTTATACCAATGTAATAAAACTATCTGTCTAATGA
1751 CTCTTGCACTGTTGCTGCCATGAAAATAGATGGCTAAATAAAAAA
GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATT
1801 GGACATTGTAATTGTAACTGAAAGGACAGCAAGTTATCGAATTCC
CCTGTAACATTAAAACATTGACTTCCCTGTCGTTCAATAAGCTTAAGG
1851 TGCAG
ACGTC

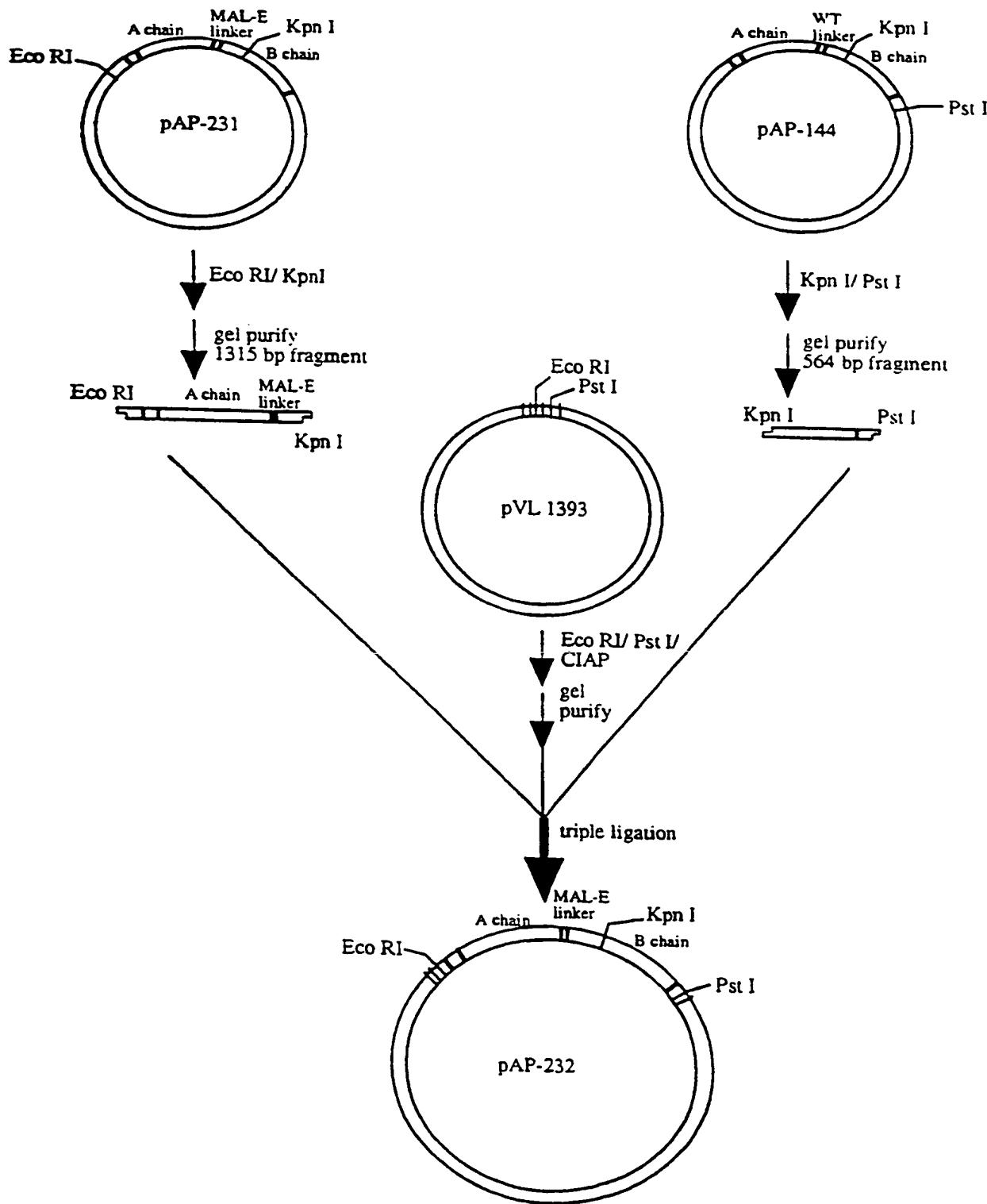
52/254

FIGURE 11A**SUBSTITUTE SHEET (RULE 26)**

53/254

FIGURE 11B**WT preprorcin linker****primer MAL-E2****pAP 231 linker
(MAL-E variant)**

54/254

FIGURE 11C**SUBSTITUTE SHEET (RULE 26)**

55/254

FIGURE 11D

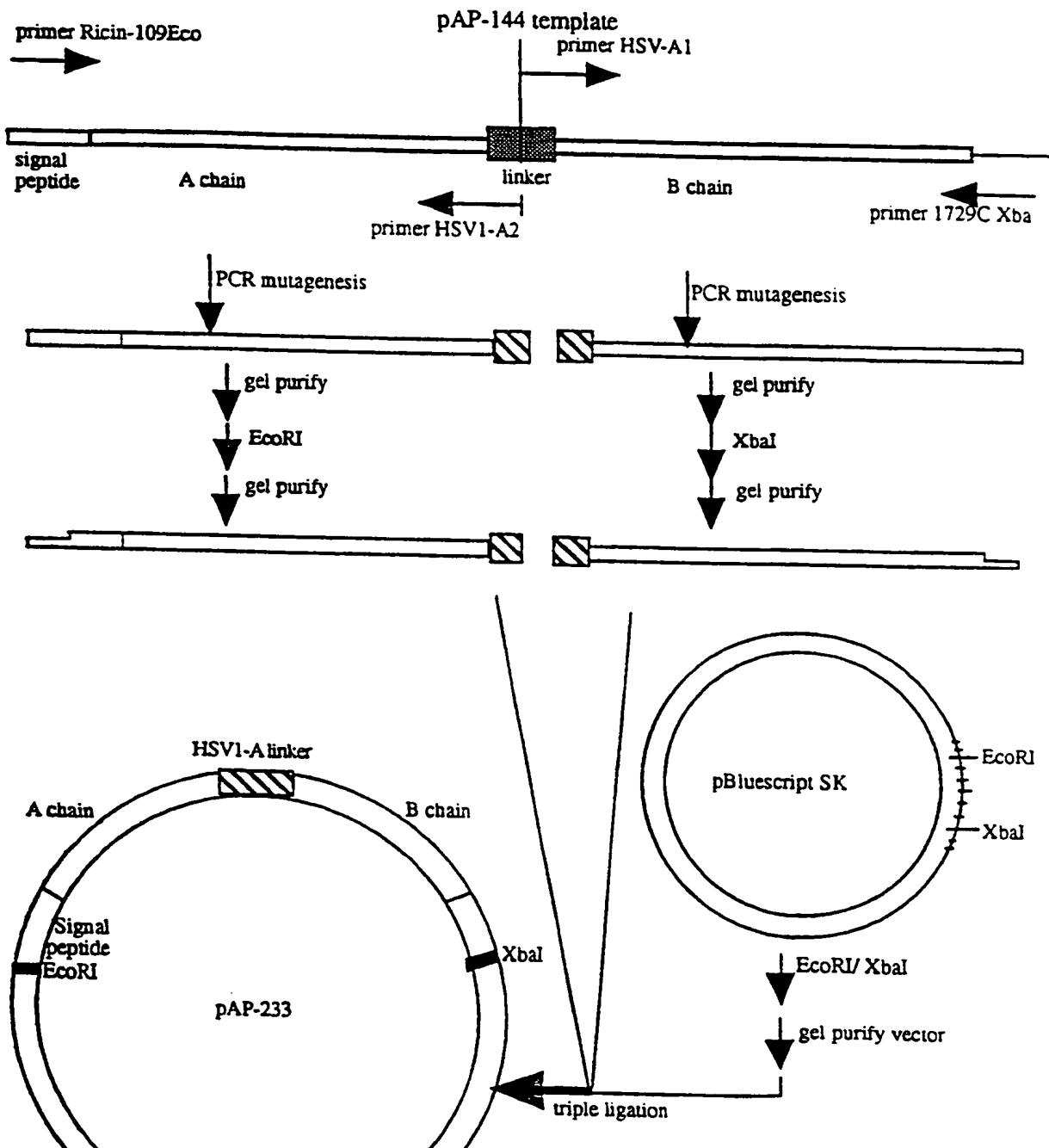
10	20	30	40	50
1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
CTTAAGTACTTTGGCCCTCTTATGATAACATTACACATACGTCA				
51 GGCAACATGGCTTTGGATCCACCTCAGGGTGGCTTTCACATTAG				
CCGTTGTACCGAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAA				
101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACCTTACCA				
TCCTATTGTTGATAAGGGGTTGTTATGGGTAATATTTGAAATGGGTG				
151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTGCCG				
CGCCCACGGTGACACGTTGATGTTGAAATAGTCGACAAGCGCC				
201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAAGTGTGCCA				
AGCAAATTGTTGACCTCGACTACACTCTGACTATATGGTCACACGGTT				
251 ACAGAGTTGGTTGCCTATAACCAACGGTTTATTTAGTTGAACCTCTCA				
TGTCCTAACCAAACGGATATTTGGTGCCTAAATAACTGAGAGT				
301 AATCATGAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
TTAGTACGTCGAAAGACAATGTAATCGCACCTACAGTGGTTACGTAT				
351 TGTGGTCGGCTACCGTGGAAATAGCGCATATTCTTCATCCTGACA				
ACACCAAGCCGATGGCACGACCTTATCGGTATAAAAGAAAGTAGGACTGT				
401 ATCAGGAAGATGAGCAATCACTCATCTTCACTGATGTTCAAAAT				
TAGTCCTCTACGTTAGTGGAGTAGAAAGTGA				
451 CGATATACTCGCTTGGTGGTAATTATGATAGACTTGAACAACCTGC				
GCTATATGTAAGCGGAAACCACCATTAATAACTATCTGAACCTGTTGAACG				
501 TGGTAATCTGAGAGAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG				
ACCATTAGACTCTTTATAGCTAACCCCTTACAGGTGATCTCCTCC				
551 CTATCTCAGCGCTTTATTACAGTACTGGTGGCACTCAGCTTCAACT				
GATAGAGTCGCAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601 CTGGCTCGTCTTATAATTGATCCAAATGATTCAGAAGCAGCAAG				
GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTTC				
651 ATTCCAATATATTGAGGGAGAAATGCGCACCGAGAATTAGGTACAACCGGA				
TAAGGTTATATAACTCCCTCTTACGCGTGTCTTACATGTTGGCCT				
701 GATCTGCACCAAGATCTAGCGTAATTACACTTGGAGAATTAGTTGGGGAGA				
CTAGACGTGGCTAGGATCGATTATGTGAACCTTATCAACCCCTCT				
751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCAAT				
GAAAGGTGACGTTAAGTTCTCAGATTGGTCTCGGAAACGATCAGGTTA				
801 TCAACTGCAAAGACGTAATGGTCCAATTCAGTGTGACGATGTGAGTA				
AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT				
851 TATTAATCCCTATCATAGCTCATGGTGTATAGATGCGCACCTCCACCA				
ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACGCGTGGAGGTGGT				
901 TCGTCACAGTTAAATTCCAAGATATGCTAAATAATTACAGCATCAGGC				
AGCAGTGTCAAATTAAAGGTTCTACGATTATTAAGTGTCTAGTCG				

56/254

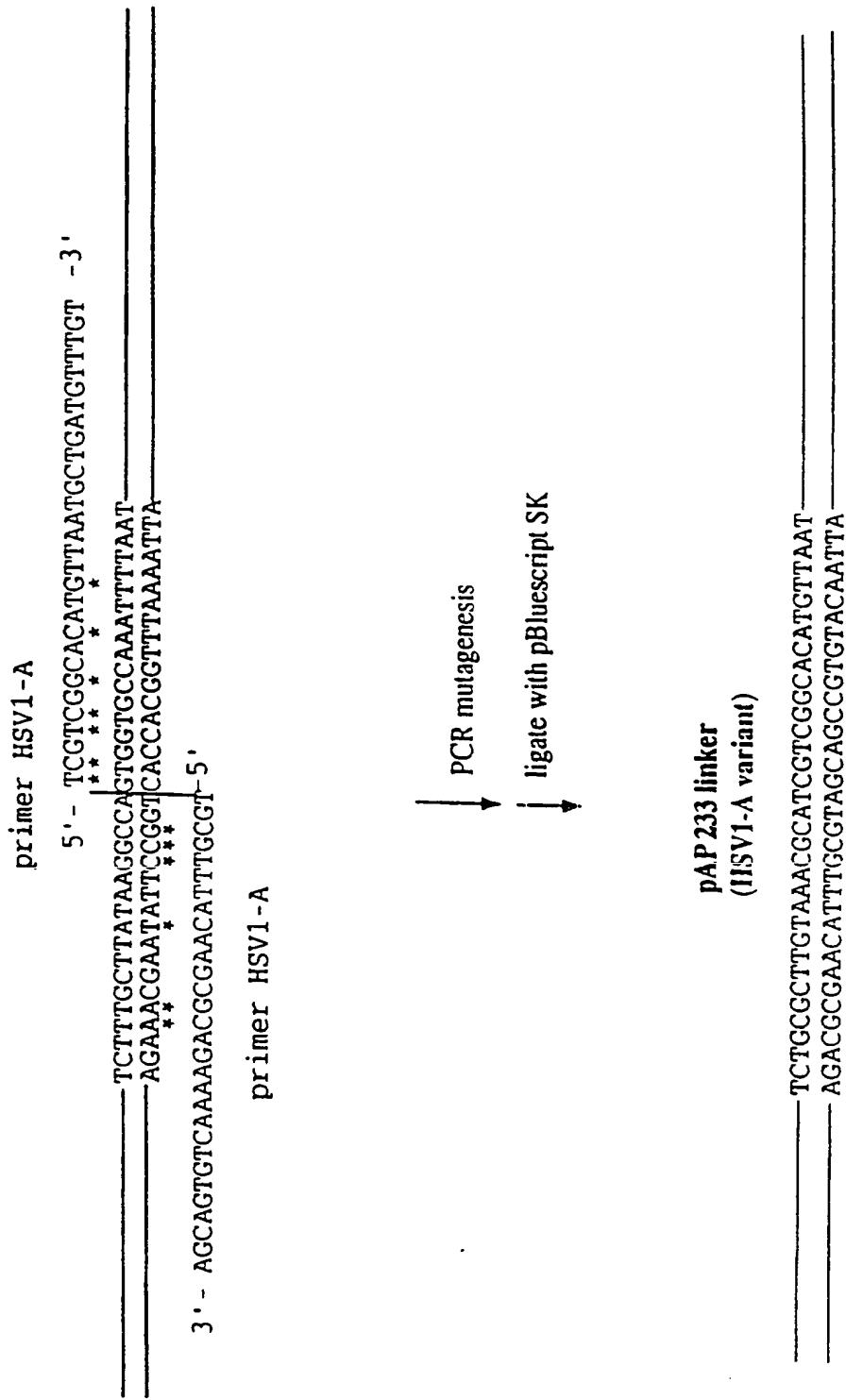
FIGURE 11D (CONT'D)

951 TGATGTTGTATGGATCCTGAGCCCATTAGTCGTATCGTAGGTCGAAATG
 ACTACAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACTACCTACCTCTAAGGTGTTGCCTTGCGTTAT
 1051 CAGTTGTGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTCAGATTATGTCAGTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAATGGAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAAACTGCTGCA
 CCATGTCAGGCCCTCAGATACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCTTACCTTGGTAGTATTAGG
 1251 CAGATCTAGTCTAGTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATAAAATCGTCGCTGAGTCCCTTGTCAACCAGGTG
 1301 TTACAGTGCAAACCAACATTATGCCGTTAGTCAAGGTTGGCTTCAACT
 AATGTCACGTTGTTGTAATAACGGCAATCAGTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG
 TTATTATGTTGAAAACAATGTTGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGA
 AACGTTGTTATCACCTGTTACACCTATCTCCTGACATCGTCACCTT
 1451 AGGCTGAACAAACAGTGGCTCTTATGCAGATGGTTCAATACTCCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCTTACAAGTGATTCTAATATAACGGGAAACAGT
 GTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTGTCA
 1551 TGTTAAGATCCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTAAATTGTATAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTTGGTAAATTAACATATCACCTAACCAACAACTCTA
 1651 GTGAGGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTACCCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAAATATGGTTACCAATTATTGTATAGACAGATTACT
 ACCACTGGGTTGGTTATACCAATGTAATAAAACTATCTGTCATAATGA
 1751 CTCTTGCACTGTGTGTCCTGCCATGAAAAATAGATGGCTAAATAAAAAA
 GAGAACGTCACACACACAGGACAGGTACTTTATCTACCGAATTATTTT
 1801 GGACATTGTAATTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAAACATTGACTTTCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

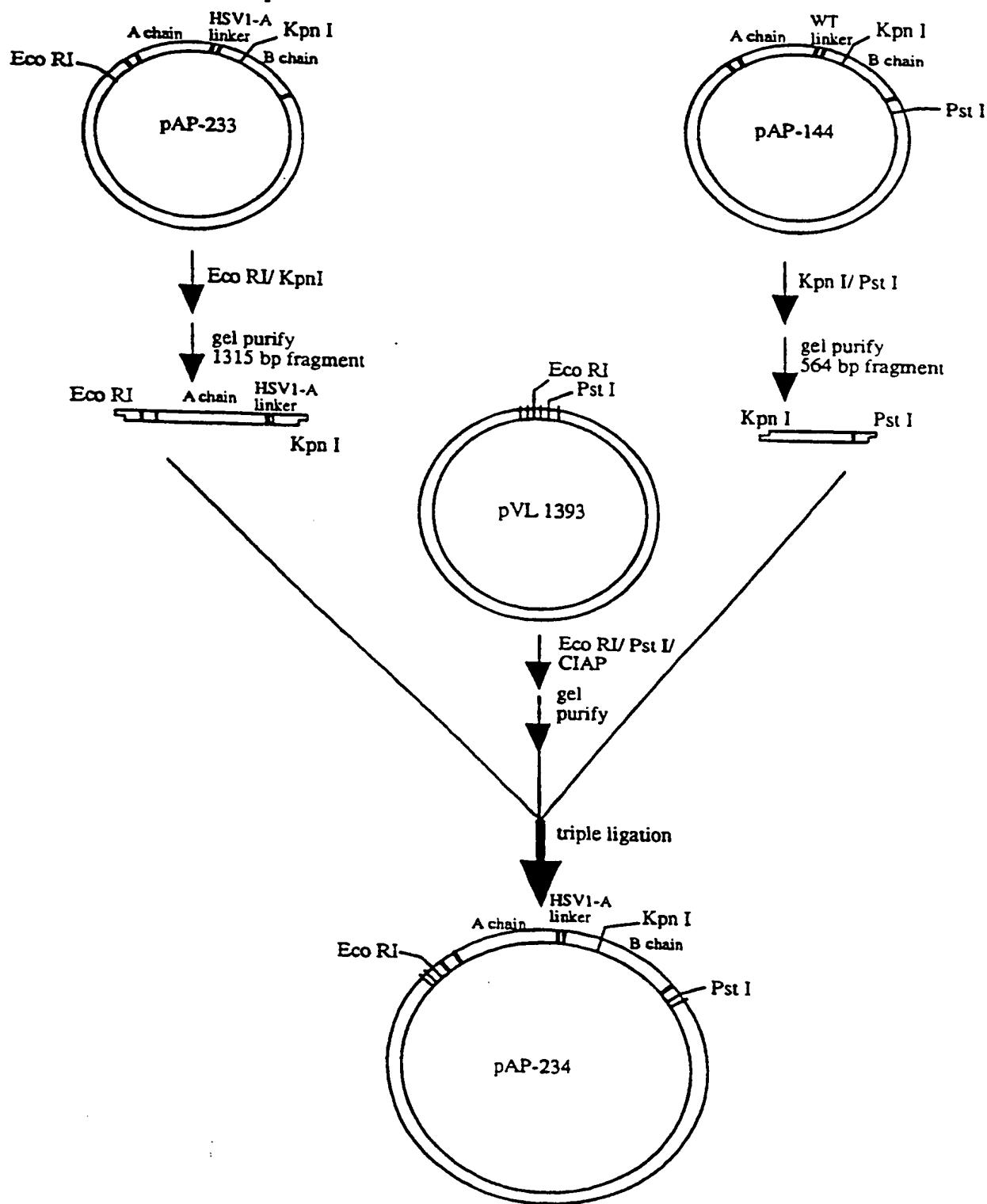
57 / 254

FIGURE 12A**SUBSTITUTE SHEET (RULE 26)**

58/254

FIGURE 12B**WT prorocin linker**

59/254

FIGURE 12C

SUBSTITUTE SHEET (RULE 26)

60/254

FIGURE 12D

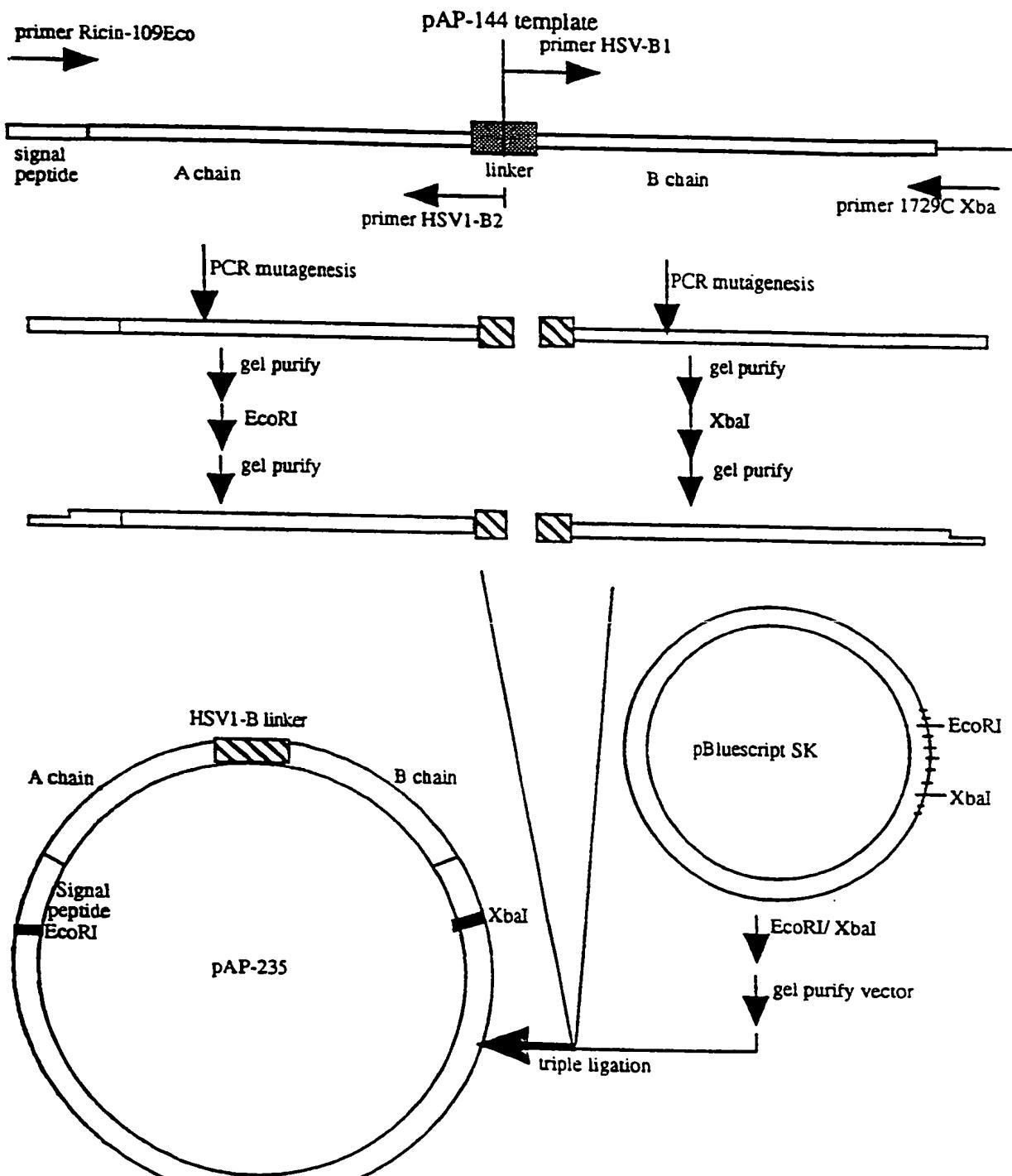
10	20	30	40	50
1 GAATT CATGAAACCGGGAGGAATACTATTGTAATATGGATGTATGCAGT				
CTTAAGTACTTTGGCCCTCCTTATGATAACATTACCTACATACGTCA				
51 GGCAACATGGCTTGTTGGATCCACCTCAGGGTGGCTTACATTAG				
CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAA				
101 AGGATAACAAACATATTCCCCAACAAACACCAATTATAAACTTTACCA				
TCCTATTGTTGATAAGGGTTTGTATGGGTTAATATTGAAATGGTGT				
151 GCGGGTGCCACTGTGCAAAGCTACACAAACCTTATCAGAGCTGTTCCGG				
CGCCCACGGTGACACGTTGATGTGTTGAAATAGTCTCGACAAGCGCC				
201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAAGTGTGCCAA				
AGCAAATTGTTGACCTGACTACACTCTGTACTATATGGTCACAACGGTT				
251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACCTCTCA				
TGTCTCAACCAAACGGATATTTGGTGCCTAACAAACTTGAGAGT				
301 AATCATGCGAGGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
TTAGTACGTCTCGAAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTCTTCATCCTGACA				
ACACCAGCCGATGGCACGACCTTATCGCGTATAAAAGAAAGTAGGACTGT				
401 ATCAGGAAGATGCGAGAACAACTCACTCATCTTCACTGATGTTCAAAAT				
TAGTCCTCTACGTCTCGTTAGTAGAAAGTGACTACAAGTTTA				
451 CGATATAACATTGCCCTTGGTGGTAATTATGATAGACTTGAACAACTTGC				
GCTATATGTAAGCGGAAACCAACCATTAATACTATCTGAACCTGTTGAACG				
501 TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG				
ACCATAGACTCTTTATAGCTAACCTTTACCGAGGTGATCTCCTCC				
551 CTATCTAGCGCTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601 CTGGCTCGTCTTATAATTGCATCCAAATGATTCAGAACAGCAAG				
GACCGAGCAAGGAAATATTAACGTAGGTTACTAAAGTCTCGTGTTC				
651 ATTCCAATATATTGAGGGAGAAAATGCGCACGAGAATTAGGTACAACCGGA				
TAAGGTTATATAACTCCCTTTACCGTGCTTTAATCCATGTTGGCCT				
701 GATCTGCACCAAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA				
CTAGACGTGGTCTAGGATCGCATTATGTGAACCTTATCAACCCCCCTCT				
751 CTTTCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT				
GAAAGGTGACGTTAAGTCTCAGATTGGTCTCGGAAACGATCAGGTTA				
801 TCAACTGCAAAGACGTAATGGTCCAATTCACTGATGTGAGTA				
AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT				
851 TATTAATCCCTATCATAGCTCTCATGGTGTATAAGATGCGCACCTCCACCA				
ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACCGTGGAGGTGGT				
901 TCGTCACAGTTTCTGCGCTTGTAAACGCATCGTCGGCACATGTTAATGC				
AGCAGTGTCAAAGACGCAACATTGCGTAGCAGCCGTGTACAATTACG				

61/254

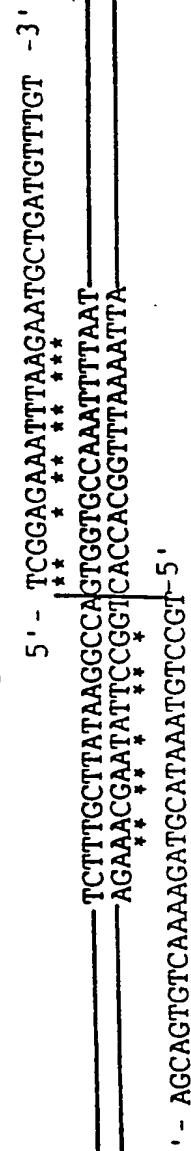
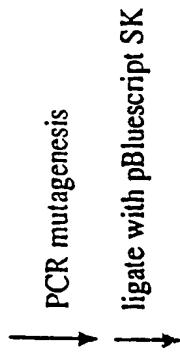
FIGURE 12D (CONT'D)

951 TGATTTGTATGGATCCTGAGCCCATAGTGCATCGTAGGTCGAATG
 ACTACAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATAATCCCTACCTCTAAGGTGTTGCCCTTGCCTTAT
 1051 CAGTTGTGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTAGATTATGCTACGTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATAACACTAGATAACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCACATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCTTATTACCTTGGTAGTATTAGG
 1251 CAGATCTAGCTAGTTTACCGAGCAGATCAGGGAAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTGTCACCATGGTGTG
 1301 TTACAGTCAAACCAACATTATGCCGTTAGTCAAGGTTGGCTTCTACT
 AATGTCACGTTGGTTGTAATAACGGAATCAGTCCAACCGAAGGATGA
 1351 AATAATACACAAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACATGTTGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGGACTGTAGCAGTGAAA
 GAACGTTCGTTTATCACCTGTCACCTATCTCCTGACATCGTCACTT
 1451 AGGCTGAACAAACAGTGGCTCTTATGCAGATGGTCAATACGTCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAAACCGAGATAATTGCCCTTACAAGTGATTCTAATATACGGAAACAGT
 GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTGTCA
 1551 TGTTAAGATCCTCTTGTGGCCCTGCATCCTCTGGCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAAATGATGGAACCATTAAATTGTTAGTGTAGGGATTGGTTAGAT
 AGTTCTTACTACCTGGTAAATTAAACATATCACCTAACACAAATCTA
 1651 GTGAGGCAGTCGGATCCGAGCCTTAAACAAATCATTCTTACCCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAAATATGGTACCAATTATTGATAGACAGATTACT
 ACCACTGGTTGGTTATACCAATGGTAATAAAACTATCTGTCTAATGAG
 1751 CTCTTGCACTGTGTCGCTGCCATGAAAATAGATGGCTAAATAAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATT
 1801 GGACATTGTAATTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTTAAACATGACTTCCCTGTCGTTCAATATAGCTTAAGG
 1851 TCCAG
 ACGTC

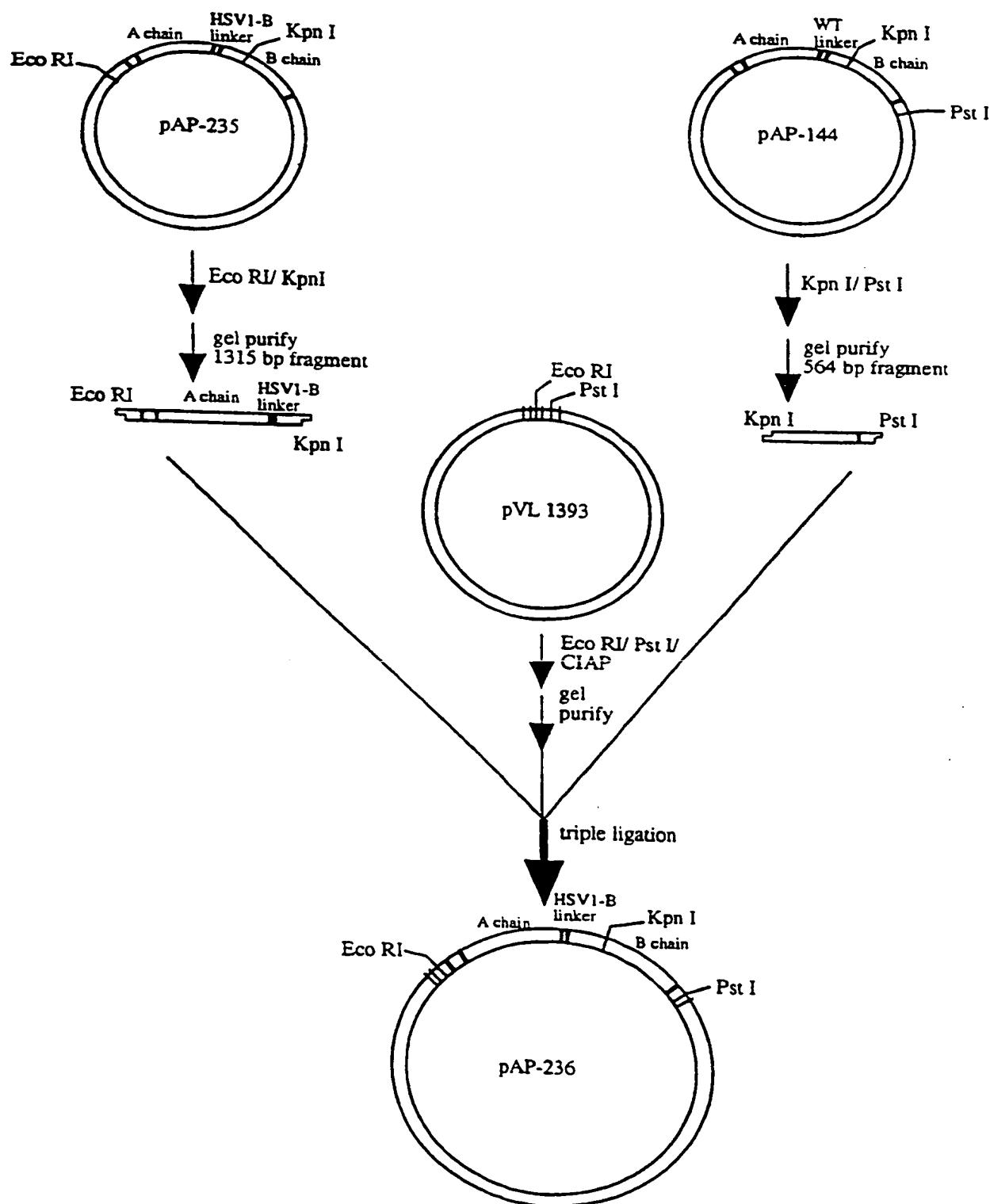
62/254

FIGURE 13A**SUBSTITUTE SHEET (RULE 26)**

63/254

FIGURE 13B**WT preprotein linker****primer HSV1-B****primer HSV1-B****pAP 235 linker
(HSV1-B variant)**

64/254

FIGURE 13C

SUBSTITUTE SHEET (RULE 26)

65/254

FIGURE 13D

10 20 30 40 50

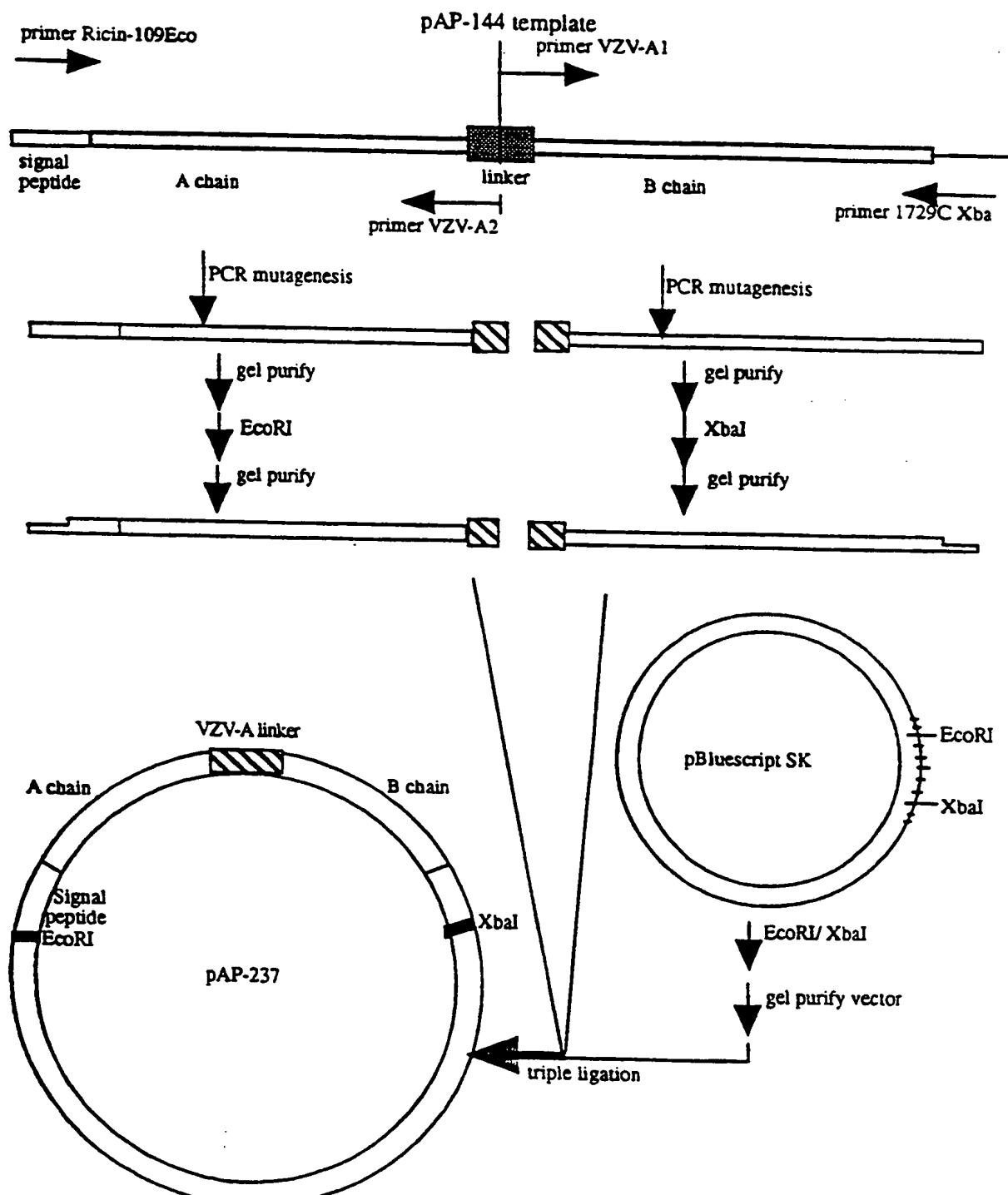
1 GAATT CATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
 CTTAAGTACTTTGCCCTCCTTATGATAACATTACCTACATACGTCA
 51 GGCAACATGGCTTGGATCCACCTCAGGGTGGTCTTCACATTAG
 CCGTTGTAACGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAA
 101 AGGATAAACACATATTCCCCAAACAATACCAATTATAAACTTTACCACA
 TCCTATTGTTGTATAAGGGTTTGTATGGTTAATATTGAAATGGTGT
 151 GCGGGTGCCACTGTGCAAAGCTACACAAACCTTATCAGAGCTGTTCGCGG
 CGCCCACGGTACACGTTCGATGTGTTGAAATAGTCTGACAAGCGCC
 201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATAACCAAGTGTGCCAA
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT
 251 ACAGAGTTGGTTGCCCTATAAACCAACGGTTTATTTAGTTGAACCTCA
 TGTCTCAACCAAACGGATATTGGTGCCTAAATAACTCAACTTGAGAGT
 301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
 TTAGTACGTCGAAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
 351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTCTTCATCCTGACA
 ACACCAAGCCGATGGCACGACCTTATCCGTATAAAGAAAGTAGGACTGT
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTCACTGATGTTCAAAAT
 TAGTCCTCTACGTCTCGTTAGTGAAGAAAGTACTACAAGTTTA
 451 CGATATACATTGCCCTTGGTGGTAATTATGATAGACTTGAACAAACTTGC
 GCTATATGTAAGCGGAAACCACCAATTAAACTATCTGAACCTGTTGAACG
 501 TCGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG
 ACCATTAGACTCTCTTATAGCTCAACCCCTTACCAAGGTGATCTCCTCC
 551 CTATCTAGCGCTTATTACAGTACTGGTGGCACTCAGCTTCCA
 GATAGAGTCGCGAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
 601 CTGGCTCGTCTTATAATTGACATCCAAATGATTCAGAACAGCAGCAAG
 GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTCGTT
 651 ATTCCAATATATTGAGGGAGAAAATGCGCACGAGAATTAGGTACAACCGGA
 TAAGGTTATATAACTCCCTCTTACCGGTGCTTTAATCCATGTTGGCCT
 701 GATCTGCACCAAGACGTAATTACACTTGAGAATAGTTGGGGAGA
 CTAGACGTGGCTAGGATCGCATTATGTAACCTTATCAACCCCTCT
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT
 GAAAGGTGACGTTAAGTCTCAGATTGGTCTCGGAAACGATCAGGTAA
 801 TCAACTGCAAAGACGTAATGGTCCAAATTCACTGAGTGTACGATGTGAGTA
 AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCATGGTGTAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACCGGTGGAGGTGG
 901 TCGTCACAGTTCTACGTATTTACAGGCATGGAGAAATTAAAGAATGC
 AGCAGTGTCAAAGATGCATAATGTCCGTAGCCTCTTAAATTCTTACG

66/254

FIGURE 13D (CONT'D)

951 TGATGTTGTATGGATCCTGAGCCCATACTGCCTAGTCAGGTCGAAATG
 ACTACAAAACATACTAGGACTCGGTATCACGCATAGCATCCAGCTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACTACAATCCCTACCTTCTAAGGTGTTGCCCTTGCCTAT
 1051 CAGTTGTGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTAGTCTACGTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAATGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
 1151 GGTACAGTCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATAACACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGCGACCCTTATACCTTACCTTGTTAGTATTAGG
 1251 CAGATCTAGTCTAGTTTAGCAGCGACATCAGGAACAGTGGTACCCAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTGTCACCATGGTGTG
 1301 TTACAGTCAAACCAACATTATGCCGTTAGTCAGGTTGGCTTCTACT
 AATGTCACGTTGGTTGTAATACGGCAATCAGTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTTGGAAAACAATGTTGTAACAACCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGGACTGTAGCAGTGA
 AACGTTCGTTATCACCTGTTACACCTATCTCTGACATCGTCACCTT
 1451 AGGCTGAACAACAGTGGGCTCTTATGCAGATGGTCAATACGTCCTCAG
 TCCGACTTGTGTCACCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCCTACAAGTGATTCTAATATACGGAAACAGT
 GTTTGGCTCTATTACCGGAATGTTCACTAAGATTATATGCCCTTGTCA
 1551 TGTTAAGATCCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTTAAATTGTATAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTTGGTAAAATTAAACATATCACCTAACCAACATCTA
 1651 GTGAGGCATGGATCCGAGCCTAAACAAATCATTCTTACCCCTCTCCA
 CACTCCGCTAGCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCAAACCAAATATGGTTACCAATTGGTGTAGACAGATTACT
 ACCACTGGGTTGGTTATACCAATGTAATAAAACATCTGTCTAATGA
 1751 CTCTTGCAGTGTGTGTCCTGCCATGAAAATAGATGGCTAAATAAAAA
 GAGAACGTACACACACAGGACGGTACTTTATCTACCGAATTATTTT
 1801 GGACATTGTAATTTGTAACTGAAAGGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAAACATTGACTTTCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

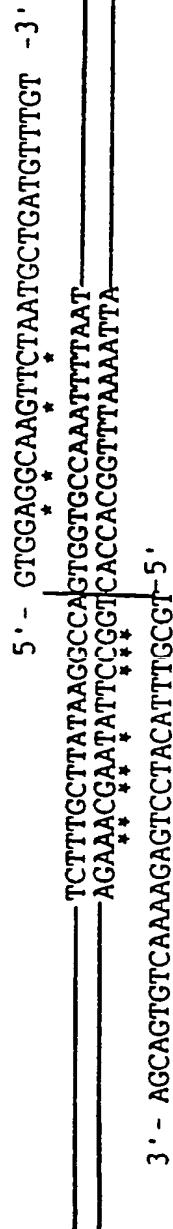
67/254

FIGURE 14A**SUBSTITUTE SHEET (RULE 26)**

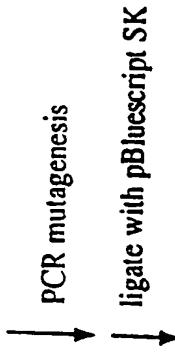
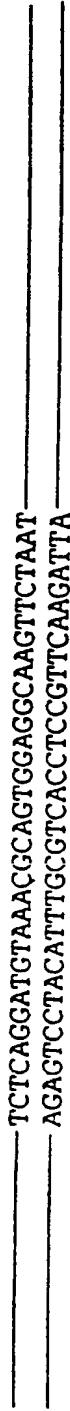
68/254

FIGURE 14B**WT preprorocin linker**

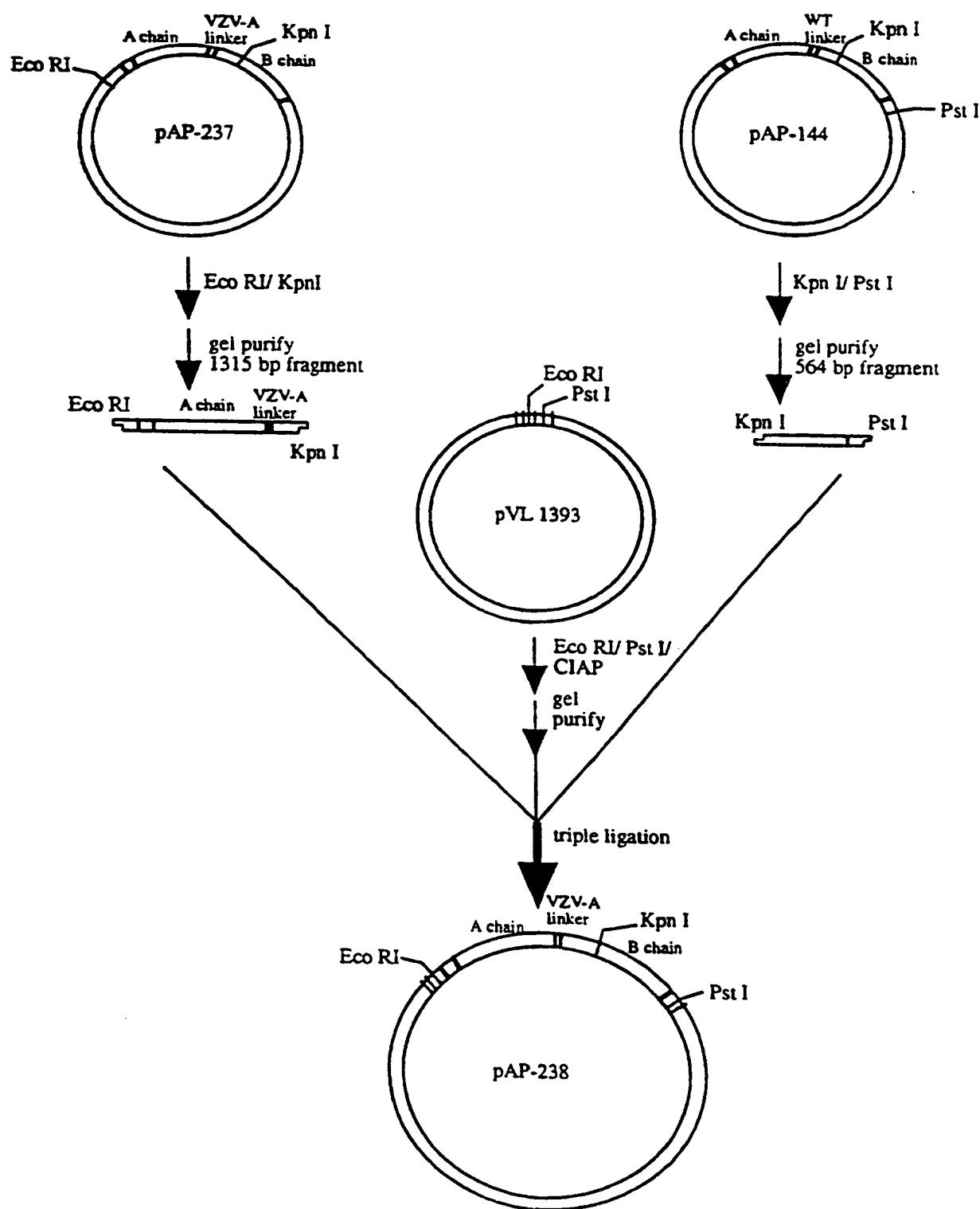
primer VZV-A1



primer VZV-A2

**pAP 237 linker
(VZV-A variant)**

69/254

FIGURE 14C

SUBSTITUTE SHEET (RULE 26)

70/254

FIGURE 14D

10	20	30	40	50
1	GAATTCATGAAACGGGAGGAAATACTATTGTAAATATGGATGTATGCAGT			
	CTTAAGTACTTTGCCCTCTTATGATAAACATTATACCTACATACGTCA			
51	GGCAACATGGCTTGTTGGATCCACCTCAGGGTGGCTTTCACATTAG			
	CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC			
101	AGGATAACAACATATTCCCCAAACAATACCCAAATTATAAACTTACACAA			
	TCCTATTGTTGATAAGGGGTTGTTATGGGTTAATATTGAAATGGTGT			
151	GCGGGTGCCACTGTGCAAAGCTACACAAAACCTTATCAGAGCTGTTGCCG			
	CGCCACGGTACACGTTGATGTTGAAATAGTCTGACAAGCGCC			
201	TCGTTTAACAACACTGGAGCTGATGTGAGACATGATATACCAAGTGTGCCAA			
	AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT			
251	ACAGAGTTGGTTGCCCTATAAACCAACGGTTATTTAGTTGAACTCTCA			
	TGTCTCAACCAACGGATATTGGTTGCCAAATAAAATCAACTTGAGAGT			
301	AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA			
	TTAGTACGTCTCGAAAGACAATGTAATCGCACCTACAGTGGTTACGTAT			
351	TGTGGTCGGCTACCGTGTGGAAATAGCGCATATTCTTACATCCTGACA			
	ACACCAGCCGATGGCACGACCTTATCGGTATAAAGAAAGTAGGACTGT			
401	ATCAGGAAGATGCAGAACATCACTCATCTTCACTGATGTTCAAAAT			
	TAGTCCTCTACGTCTCGTTAGTAGAAAGTAGACTACAAGTTTA			
451	CGATATACATTGCCCTTGGTGTAAATTATGATAGACTTGAACAACTTGC			
	GCTATATGTAAGCGAACCCACCATTAATAACTATCTGAACCTGTTGAACG			
501	TGGTAATCTGAGAGAAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG			
	ACCATTAGACTCTTTATAGCTAACCCCTTACCAAGGTGATCTCCCTCC			
551	CTATCTAGCCCTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC			
	GATAGAGTCGCGAAATAATAATGTATGACCGTGAGTCGAAGGTTGA			
601	CTGGCTCGTCTTATAATTGACATCCAAATGATTCAGAACAGCAAG			
	GACCGAGCAAGGAAATATAACGTAGGTTACTAAAGTCTCGTCGTC			
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA			
	TAAGGTTATATAACTCCCTTTACCGTGTCTTAATCCATGTTGGCCT			
701	GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAAATAGTTGGGGAGA			
	CTAGACGTGGCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT			
751	CTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT			
	GAAAGGTGACGTTAAGTCTCAGATTGGTCCCTCGAAACGATCAGGTAA			
801	TCAACTGCAAAGACGTAATGGTCAAATTCACTGAGTACGATGTGAGTA			
	AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT			
851	TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCCACCTCCACCA			
	ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACCGTGGAGGTGGT			
901	TCGTACACAGTTCTCAGGATGTAACGCAGTGGAGGCAAGTTCTAATGC			
	AGCAGTGTAAAAGAGTCCATATTGCGTACCTCCGTTCAAGATTACG			

71/254

FIGURE 14D (CONT'D)

951 TGATGTTGTATGGATCCTGAGCCCATA GTGCGTATCGTAGGTCGAAATG
 ACTACAAACATA CCTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC

 1001 GCTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACTACAATCCCTACCTTCTAAGGTGTTGCCTTGCGTTAT

 1051 CAGTTGTGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTCAAGATTATGCTACGTTAGTCAGACCTGAAA

 1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC

 1151 GGTACAGTCCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCTTATTACCTTGGTAGTATTAGG

 1251 CAGATCTAGTCTAGTTTAGCAGCGACATCAGGGAACAGTGGTACCCACAC
 GTCTAGATCAGATCAAATCGTCGCTGAGTCCCTGTCACCATGGTGTG

 1301 TTACAGTGCACACCAACATTATGCCCTAGTCAAGGTTGGCTTCTACT
 AATGTCACGTTGGTTGTAATACGGCAATCAGTTCCAACCGAAGGATGA

 1351 AATAATACACACACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTTGGAAAACAATGTTGTAACACCCGATATACCAAGACAC

 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGA
 AAACGTTCTACCTACCTATCTCCTGACATCGTCACCTT

 1451 AGGCTGAACAAACAGTGGCTCTTATGCAGATGGTCAATACGTCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

 1501 CAAACCGAGATAATTGCCCTACAAGTGATTCTAATATACGGAAACAGT
 GTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTGTC

 1551 TGTTAAGATCCTCTTGTTGGCCCTGCATCCTCTGGCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

 1601 TCAAGAATGATGGAACCATTAAATTGTTAGTATGTTAGGATTGGTGTAGAT
 AGTTCTTACTACCTGGTAAATTAACATATCACCTAACCAATCTA

 1651 GTGAGGCATGGATCCGAGCCTTAAACAAATCATTCTTACCCCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT

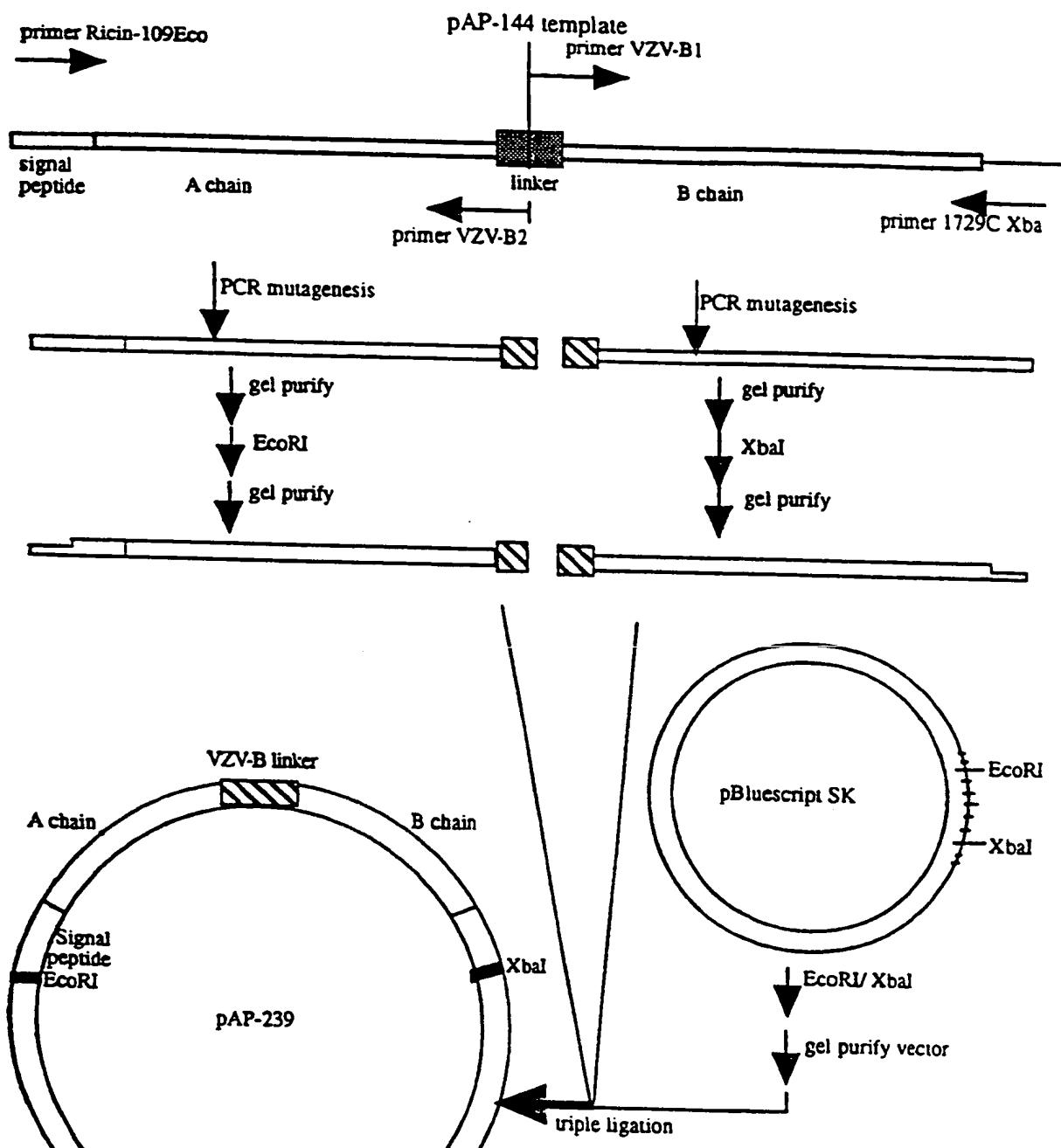
 1701 TGGTACCCAAACCAAATATGGTACCAATTATTGATAGACAGATTACT
 ACCACTGGGTTGGTTATACCAATGGAATAAAACTATCTGTCTAATGA

 1751 CTCTTGCAGTGTGTGTCCTGCCATGAAAATAGATGGCTAAATAAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTTT

 1801 GGACATTGTAATTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAAACATTGACTTCCCTGCGTTCAATATAGCTTAAGG

 1851 TGCAG
 ACGTC

72/254

FIGURE 15A

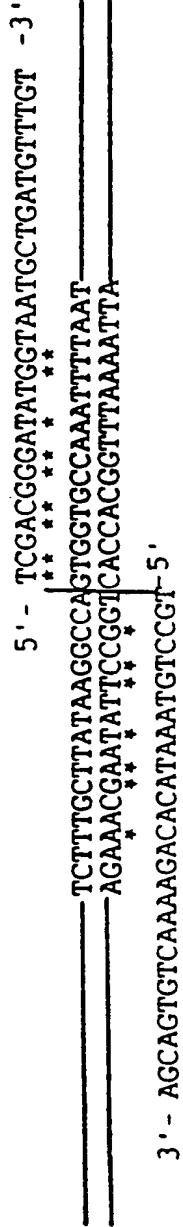
SUBSTITUTE SHEET (RULE 26)

73/254

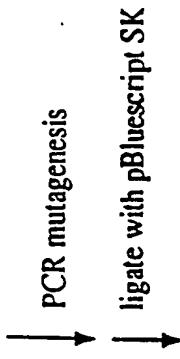
FIGURE 15B

WT preprorcin linker

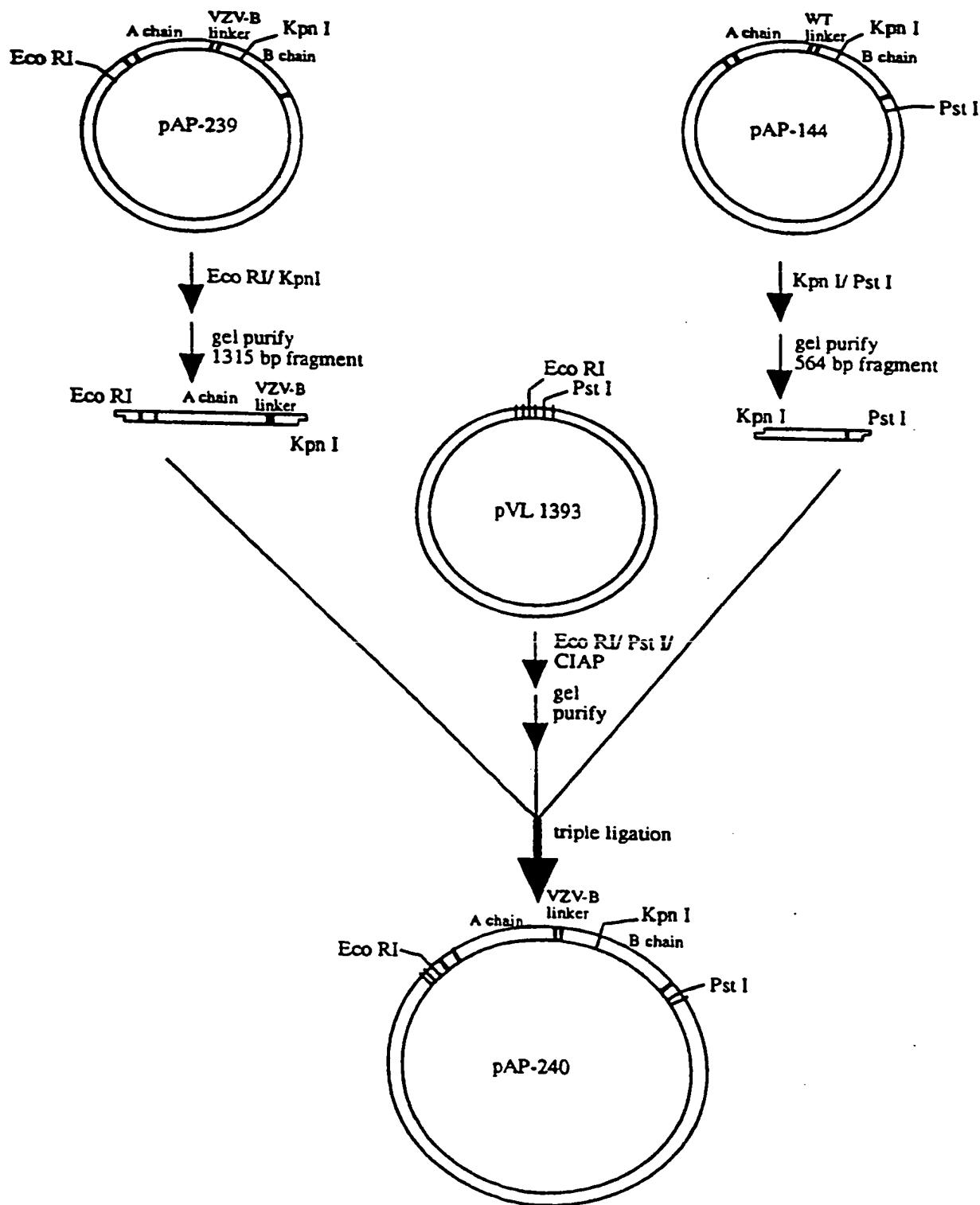
primer VZV-B1



primer VZV-A2

pAP239 linker
(VZV-B variant)

74/254

FIGURE 15C**SUBSTITUTE SHEET (RULE 26)**

75/254

FIGURE 15D

10	20	30	40	50
1 GAATTCATGAAACCGGGAGGAATACTATTGTAATATGGATGTATGCAGT CTTAAGTACTTGGCCCTCCTTATGATAACATTACACATACGTCA				
51 GGCAACATGGCTTGTGAGACATGATGAGACATGATGATACCAAGTGGTCTTCACATTAG CCGTTGTAACCGAAAACAAACCTAGGTGGAGTCCCACCAGAAAAGTGTAAATC				
101 AGGATAACAACATATTCCCCAACAAACAATACCAATTATAAACTTTACCA TCCTATTGTTGATAAGGGTTGTTATGGGTTAATATTGAAATGGTGT				
151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTGTTCGCG CGCCCACGGTGACACGTTGATGTGTTGAAATAGTCTCGACAAGCGCC				
201 TCGTTAACAACTGGAGCTGATGTGAGACATGATGATACCAAGTGGTCCAA AGCAAATTGTTGACCTCGACTACACTCTGTAATATGGTCACAACGGTT				
251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACTCTCA TGTCTCAACCAAACGGATATTGGTTGCCAAATAAAATCAACTTGAGAGT				
301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTACCCAATGCATA TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351 TGTGGTCGGTACCGTGCTGGAAAATAGCGCATATTCTTCATCTGACA ACACCAGCCGATGGCACGACCTTATCGCGTATAAGAAAGTAGGACTGT				
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTCACTGATGTTAAAAT TAGTCCTCTACGTCTCGTTAGTAGAAAGTAGACTACAAGTTTA				
451 CGATATACTCGCTTTGGGTAAATTATGATAGACTTGAACAACCTGC GCTATATGTAAGCGGAAACCAACCATTAATACTATCTGAACTTGTGAACG				
501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCACTAGAGGAGG ACCATTAGACTCTCTTATAGCTCAACCCCTTACCAAGGTGATCTCCTCC				
551 CTATCTCAGCGCTTTATTACAGTACTGGTGGCACTCAGCTTCAA GATAGAGTCGCAAATAATGTATGACCGTACCGTGAAGTTGAAGGTTGA				
601 CTGGCTCGTCTTTATAATTGATCCAAATGATTTCAAGCAGCAAG GACCGAGCAAGAAAATATTAACGTAGGTTACTAAAGTCTCGTGTTC				
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA TAAGGTTATATAACTCCCTCTTACCGCGTCTTAATCCATGTTGGCCT				
701 GATCTGCACCAAGATCCTAGCGTAATTACACTTGAAGAATAGTTGGGGAGA CTAGACGTGGTCTAGGATCGCATTATGTGAACCTTATCAACCCCTCT				
751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT GAAAGGTGACGTTAAGTCTCAGATTGGTCTCGGAAACGATCAGGTTA				
801 TCAACTGCAAAGACGTAATGGTCAAATTCAAGTGTACGATGTGAGTA AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT				
851 TATTAATCCCTATCATAGCTCTCATGGTGATAGATGCGCACCTCCACCA ATAATTAGGGATAGTATCGAGAGTACCCACATATCTACCGTGGAGGTGGT				
901 TCGTCACAGTTCTGTGTTACAGGCATCGACGGGATATGGTAATGC AGCAGTGTCAAAGACACATAATGTCCGTAGCTGCCCTATACCATTACG				

76/254

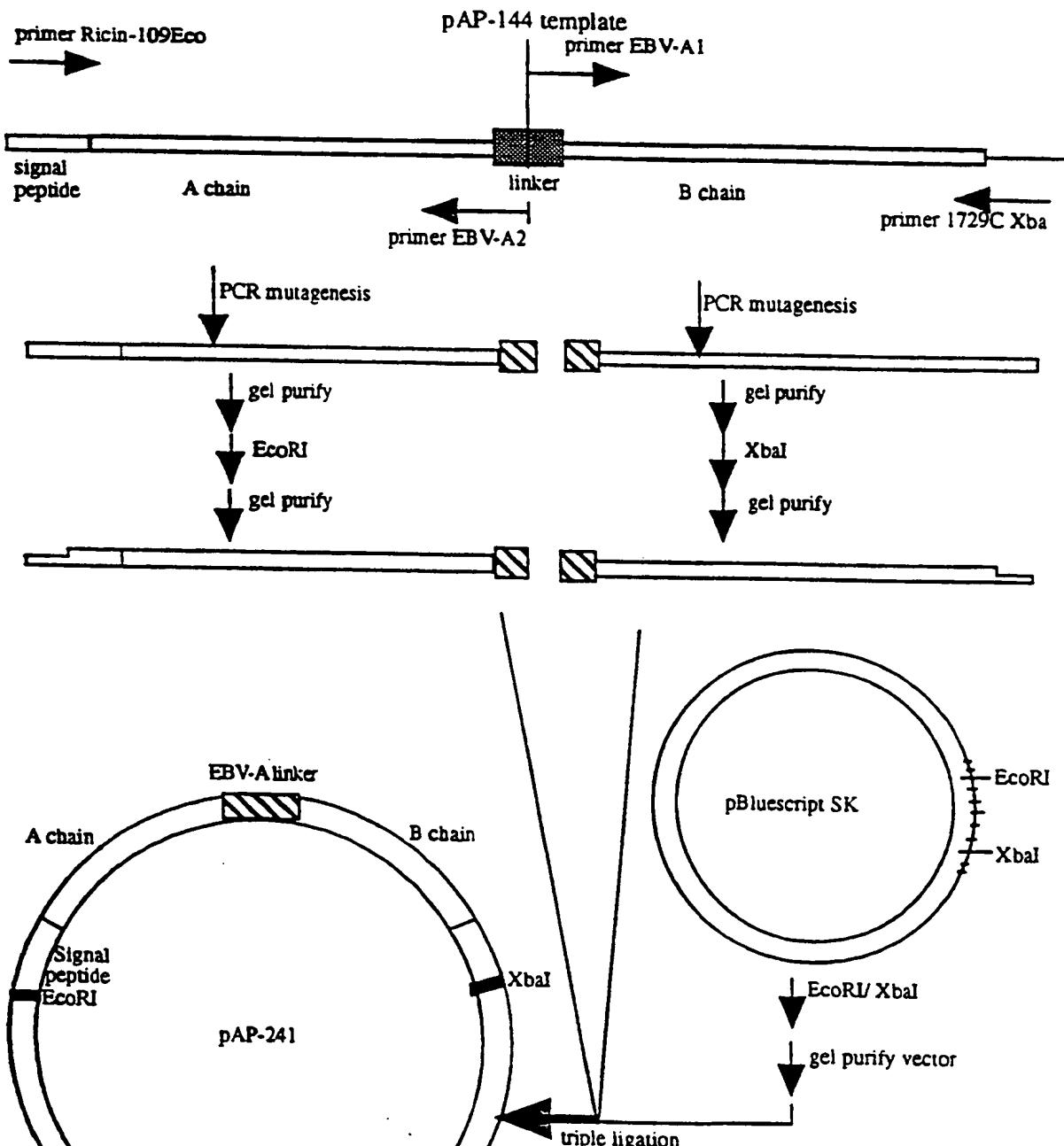
FIGURE 15D (CONT'D)

951 TGATGTTGTATGGATCCTGAGCCCATA GTGCGTATCGTAGGTCGAAATG
 ACTACAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACTACATCCCTACCTCTAAGGTGTTGCCCTTGCCTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTCAAGATTATGTCTACGTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAATGGAAGTGTTAACTACTTACG
 CTTTCTCTGTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAAACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTAGATAACTAACGTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCACATCATAAATCC
 TGAATACGGTGGCGACCGTTATACCCATTACCTTGGTAGTATTAGG
 1251 CAGATCTAGTCTAGTTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCTTGTCAACCATGGTGTG
 1301 TTACAGTCAAACCAACATTATGCCGTTAGTCAGGTTGGCTTCCTACT
 AATGTCACGTTGGTTGTAACATGCCAATCAGTTCAACCGAAGGATGA
 1351 AATAATACACAAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATATGTGTTGGAAAACAATGTTGGTAACAACCGATATACCAAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGA
 AACGTTCGTTATCACCTGTTACACCTATCTCCTGACATCGTCACTT
 1451 AGGCTGAACAAACAGTGGCTTTATGCAGATGGTCAATACGTCCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCCTTACAAGTGATTCTAATATACGGAAACAGT
 GTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTGTCA
 1551 TGTTAAGATCCTCTTGTGGCCCTGCATCCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTAAATTGTTAGTGATAGGGATGGTGTAGAT
 AGTTCTTACTACCTTGGTAAATTTAAACATATCACCTAACCAACAACTCA
 1651 GTGAGGCGATCGGATCCCGAGCCTTAAACAAATCATTCTTACCCCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTGATAGACAGATTACT
 ACCACTGGGTTGGTTATACCAATGGAATAAAACTATCTGTCTAATGA
 1751 CTCTTGCAGTGTGTGTCCTGCCATGAAAAATAGATGGCTAAATAAAAA
 GAGAACGTACACACACAGGACGGTACTTTATCTACCGAATTATTTT
 1801 GGACATTGTAATTGTAACGTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAACATTGACTTCCCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

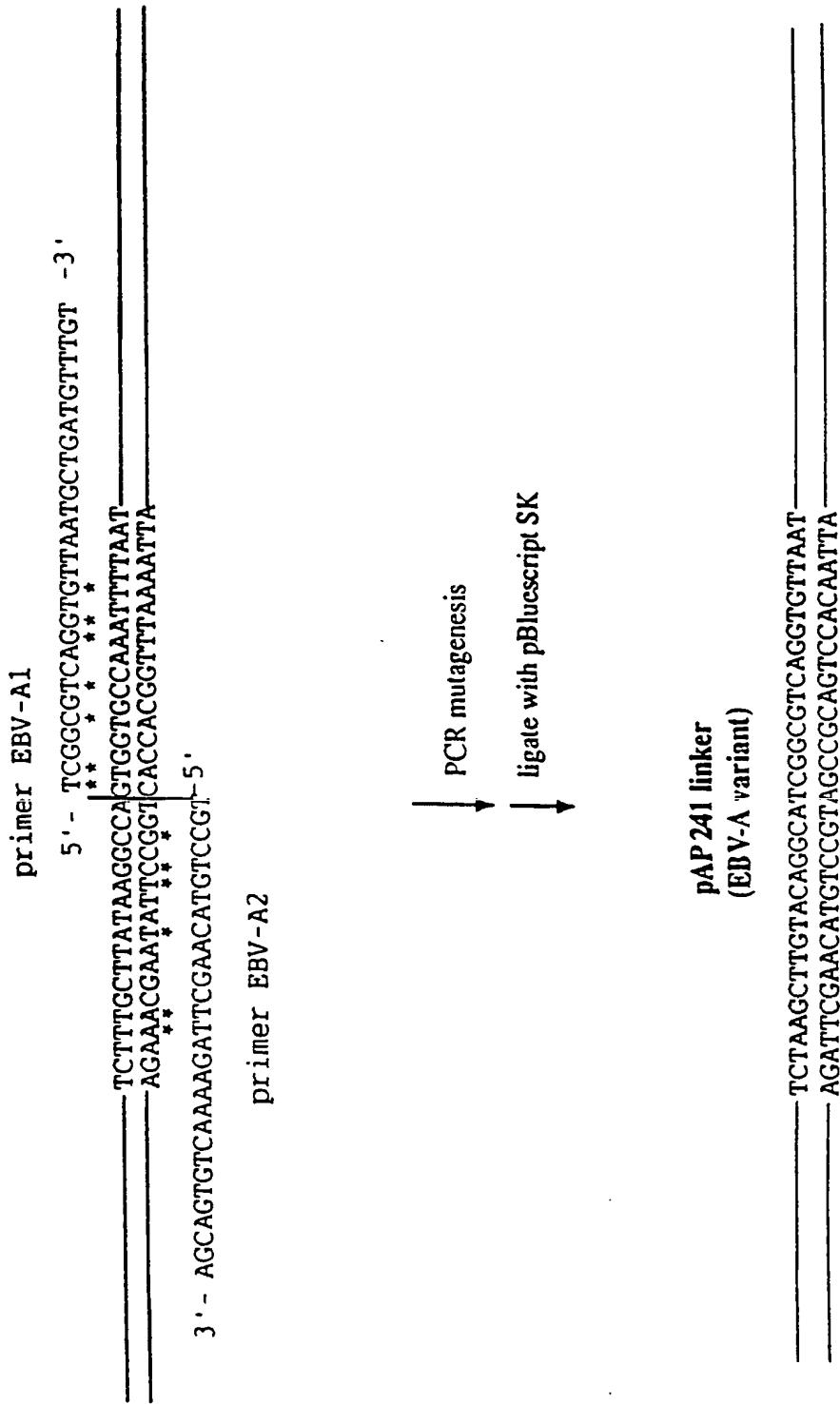
77/254

FIGURE 16A

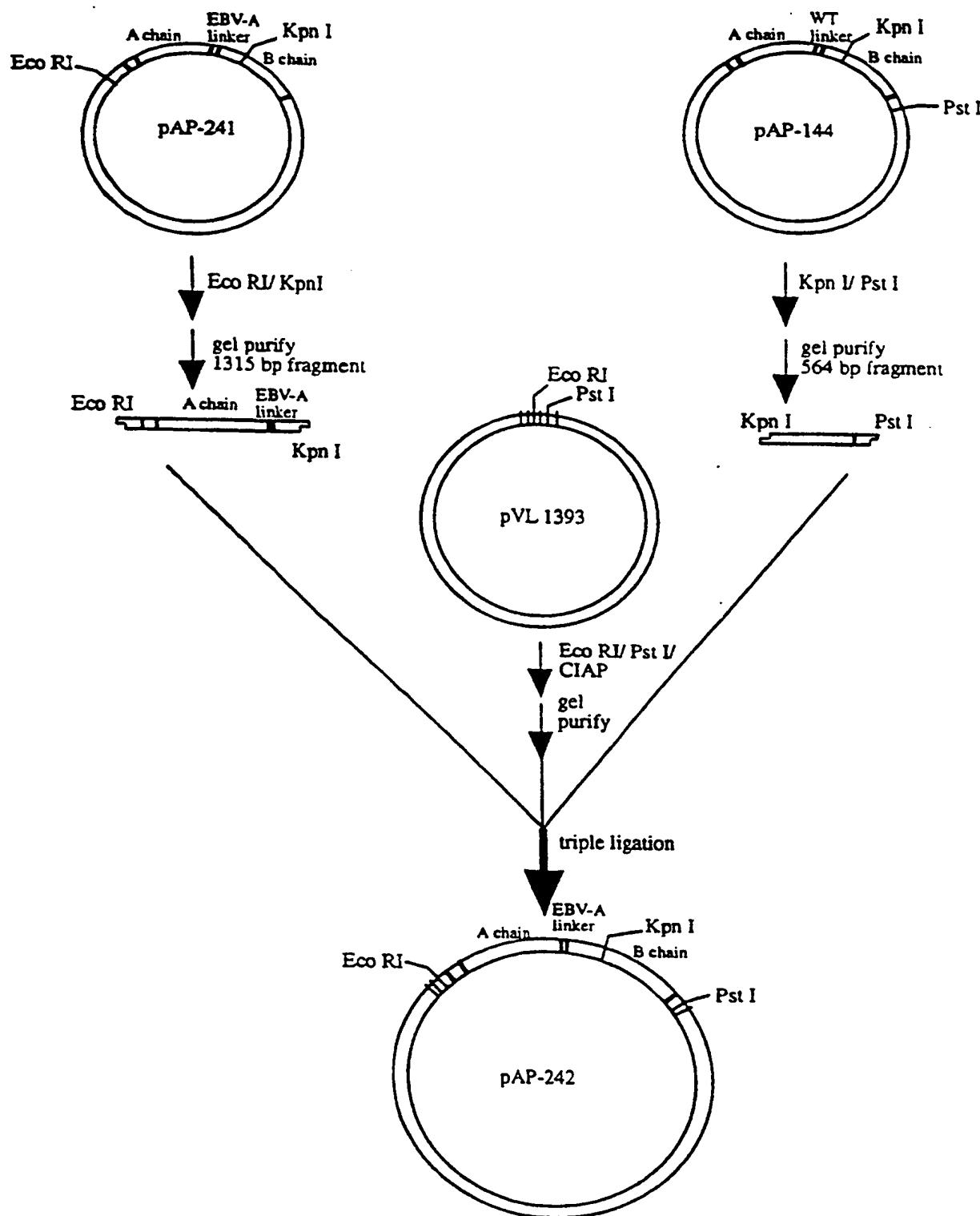
**PCR Mutagenesis of Preproricin Gene to Create an EBV-A Variant
Gene a) Cloning Strategy**

**SUBSTITUTE SHEET (RULE 26)**

78/254

FIGURE 16B**WT preprorocin linker**

79/254

FIGURE 16C

SUBSTITUTE SHEET (RULE 26)

80/254

FIGURE 16D

10	20	30	40	50
1 GAATTCA	GAACCGGGAGGAAATAC	TATGTAATATGGATGTATGCAGT		
	CTTAAGTACTTGGCCCTCTTATGATAACATTATAACCTACATACGTCA			
51 GGCAACATGGCTTGTGATCCACCTCAGGGTGGCTTTCACATTAG				
	CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAA			
101 AGGATAACAACATATTCCCCAAACAATACCCATTATAAACTTACCA				
	TCCTATTGTTGATAAGGGTTGTATGGTTAATATTGAAATGGTGT			
151 GCGGGTGCCACTGTGCAAAGCTACACAAACCTTATCAGAGCTGTCGCGG				
	CGCCACGGTGACACGTTGATGTGTTGAAATAGTCTCGACAAGCGCC			
201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAAGTGTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGACTATATGGTCACAACGGTT			
251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACTCTCA				
	TGTCTCAACCAACGGATATTGGTTGCCAAATAAAATCAACCTGAGAGT			
301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCACCTACAGTGGTTACGTAT			
351 TGTGGTCGGCTACCGTGTGGAAATAGCGCATATTCTTCATCCTGACA				
	ACACCAAGCCGATGGCACGACCTTATCGCGTATAAGAAAGTAGGACTGT			
401 ATCAGGAAGATGCAGAACAACTCACTCATCTTCACTGATGTTCAAAAT				
	TAGTCCTTCTACGTCTCGTTAGTAGAAAGTGAACACTAAGTTTA			
451 CGATATACATTGCCCTTGGTGTAAATTATGATAGACTTGAACAAACTTGC				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG			
501 TGGTAATCTGAGAGAAAAATATGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTATAGCTAACCCCTTACCAAGGTGATCTCCCTCC			
551 CTATCTCAGCGCTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA			
601 CTGGCTCGTTCTTATAATTGCA	TCACCAATGATTTCAGAACAGCAGCAAG			
	GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTCGTTC			
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTACCGTGTCTTAATCCATGTTGGCCT			
701 GATCTGACCAAGATCTAGCGTAATTACACTTGAGAAATAGTTGGGGAGA				
	CTAGACGTGGTCTAGGATCGCATTATGTAACCTTATCAACCCCTCT			
751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT				
	GAAAGGTGACGTTAAGTCTCAGATTGGTCCCTCGGAAACGATCAGGTTA			
801 TCAACTGCAAAGACGTAAAGGTCAAATTCAAGGTGTACGATGTGAGTA				
	AGTTGACGTTCTGCATTACCAAGGTTAACGTACACATGCTACACTCAT			
851 TATTAATCCCTATCATAGCTCTATGGGTATAGATGCGCACCTCCACCA				
	ATAATTAGGGATAGTATCGAGAGTACCCACATATCTACCGTGGAGGTGGT			
901 TCGTCACAGTTTCTAAGCTTGTACAGGCATCGCGTCAGGTGTTAATGC				
	AGCAGTGTCAAAGATTGAAACATGTCGTAAGCCGAGTCCACAATTACG			

81/254

FIGURE 16D (CONT'D)

951 TGATTTGTATGGATCCTGAGCCCATACTGGTATCGTAGGTGAAATG
 ACTACAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC

 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGAAACGCAATA
 CAGATACACAACATACTACCTACCTCTAAGGTGTTGCCCTTGCCTTAT

 1051 CAGTTGTGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTAGATTATGTCAGTTAGTCGAGACCTGAAA

 1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC

 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTAGATACTAACGTTATGACGACGT

 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCCATTACCTTGGTAGTATTAGG

 1251 CAGATCTAGTCTAGTTTAGCAGCGACATCAGGGAACAGTGGTACACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCTTGTACCATGGTGTG

 1301 TTACAGTGCAAACCAACATTATGCCGTTAGTCAGGTTGGCTTCACT
 AATGTCACGTTGGTTGAAATACGGCAATCAGTCCAACCGAAGGATGA

 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

 1401 CTTGCAAGCAAATAGGGACAAGTATGGATAGAGGACTGTAGCAGTGGAAA
 GAACGTTGTTATCACCTGTTACACCTATCTCTGACATCGTCACTT

 1451 AGGCTGAACAAACAGTGGCTTTATGCAGATGGTCAATACTGCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

 1501 CAAAACGAGATAATTGCCCTACAAGTGATTCTAATATACGGAAACAGT
 GTTTGGCTCTATTAACGGAATGTTACTAAGATTATATGCCCTTGTCA

 1551 TGTTAAGATCCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCCGGACGTAGGAGACCGGTTGCTACCTACA

 1601 TCAAGAATGAGGAAACCATTAAATTGTTAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTGGTAAATTAAACATATCACCTAACCAATCTA

 1651 GTGAGGCGATCGGATCCGAGCCTAAACAAATCATTCTTACCCCTCTCCA
 CACTCCGCTAGGCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT

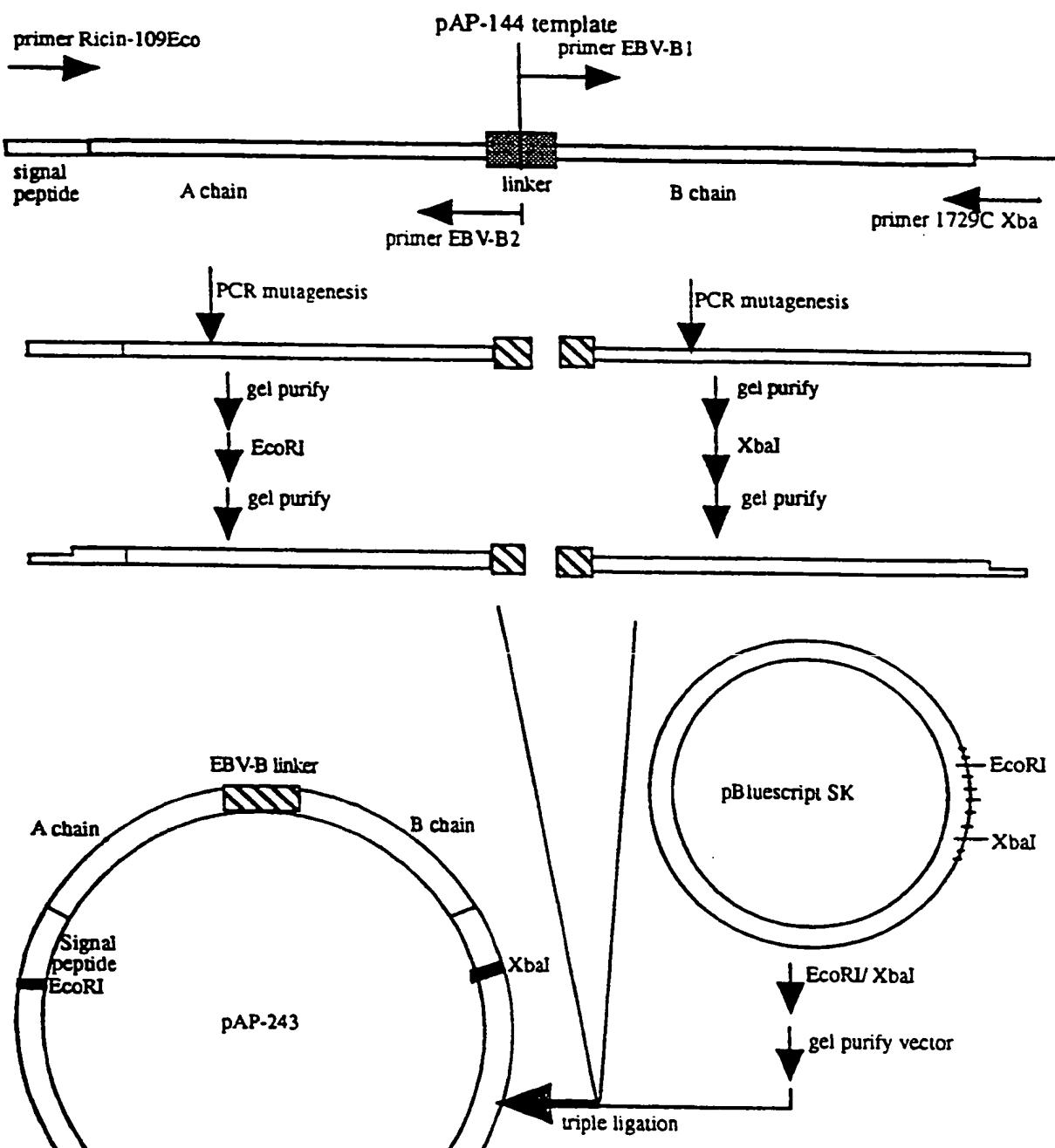
 1701 TGGTACCCAAACAAATATGGTTACCATTATTTGATAGACAGATTACT
 ACCACTGGGTTGGTTATACCAATGGTAATAAAACTATCTGTCTAATGA

 1751 CTCTTGCAGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTT

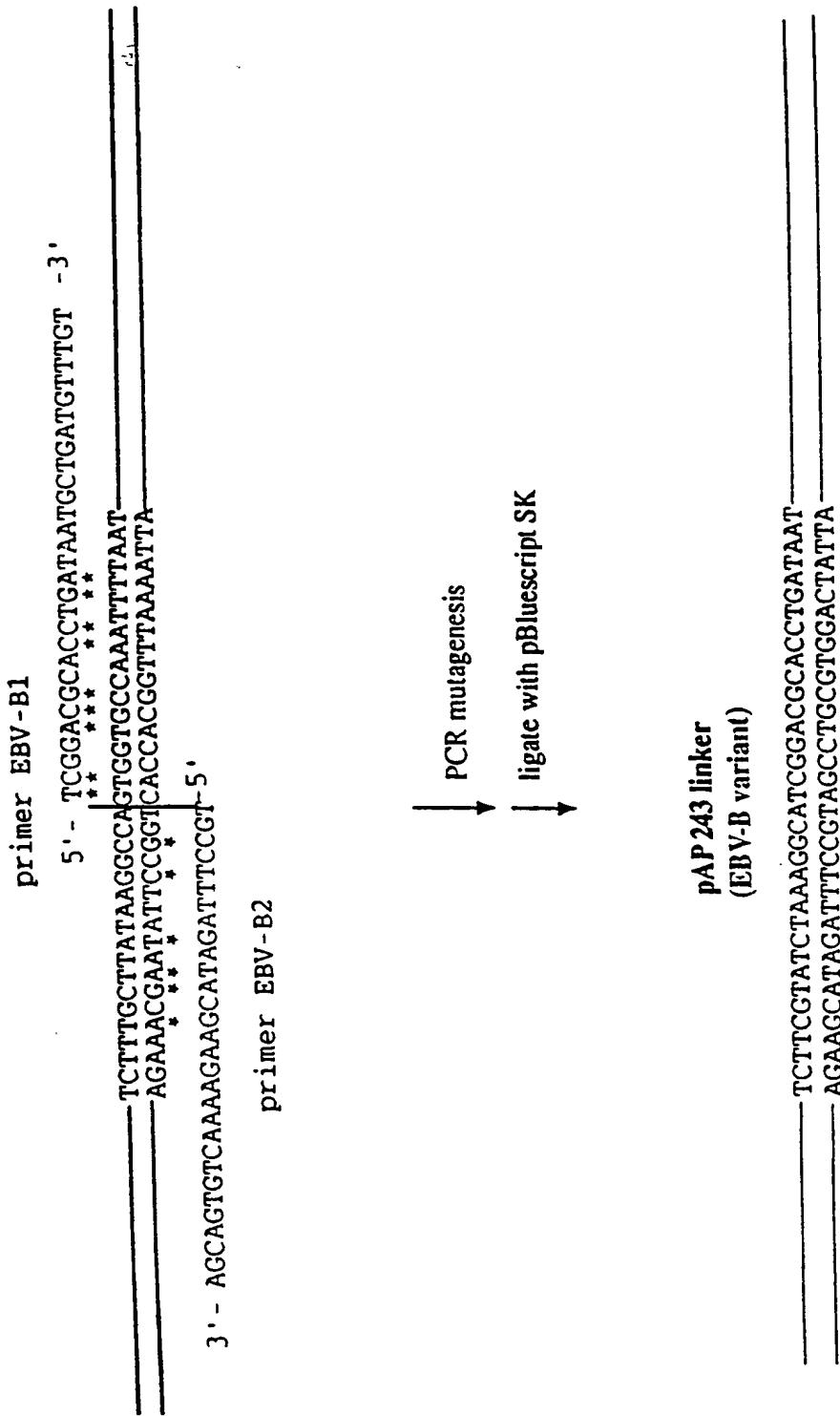
 1801 GGACATTGTAATTGTAACGTAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAAACATTGACTTCTGTCGTTCAATATAGCTTAAGG

 1851 TGCAG
 ACGTC

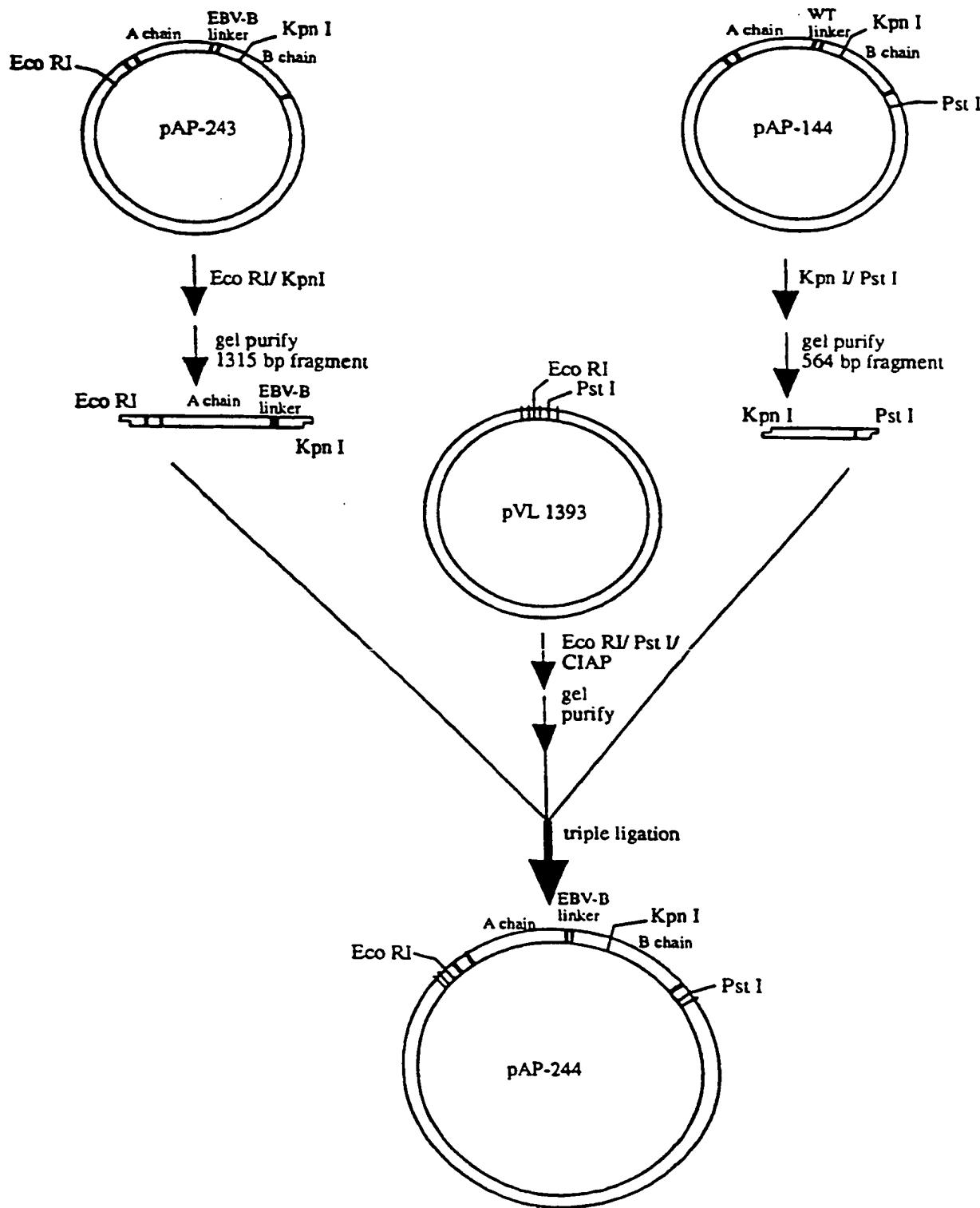
82/254

FIGURE 17A**SUBSTITUTE SHEET (RULE 26)**

83/254

FIGURE 17B**WT preorotic linker**

84/254

FIGURE 17C

85/254

FIGURE 17D

10	20	30	40	50
1 GAATTCATGAAACGGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
CTTAAGTACTTTGGCCCTCTTATGATAACATTATACCTACATACGTCA				
51 GGCAACATGGCTTGTTGGATCCACCTCAGGGTGGCTTTCACATTAG				
CCGTTGTACCGAAAACAAACCTAGGTGGAGTCCCACCAGAAAAGTGTAA				
101 AGGATAACAACATATTCCCCAACAAACACCAATTATAAACTTACCA				
TCCTATTGTTGATAAGGGGTTGTTATGGGTTAATATTGAAATGGTGT				
151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTGTCGCC				
CGCCACGGTGACACGTTGATGTGTTGAAATAGTCTCGACAAGCGCC				
201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAAGTGTGCCA				
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT				
251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACCTCTCA				
TGTCTCAACCAACGGATATTGGTTGCCAAATAAAATCAACTTGAGAGT				
301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCAACCAATGCATA				
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351 TGTGGTCGGCTACCGTGCCTGGAAATAGCGCATATTCTTCATCCTGACA				
ACACCAGCGATGGCACCGACCTTATCGGTATAAAGAAAGTAGGACTGT				
401 ATCAGGAAGATGCAGAACATCACTCATCTTCACTGATGTTCAAAAT				
TAGTCCTCTACGTCTCGTTAGTAGAAAGTAGACTACAAGTTTA				
451 CGATATACATTGCCCTTGGTGGTAATTATGATAGACTTGAACAACTTGC				
GCTATATGTAAGCGAACCCACCATTAATACTATCTGAACCTGTTGAACG				
501 TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG				
ACCATTAGACTCTCTTTATAGCTAACCCCTTACAGGTGATCTCCTCC				
551 CTATCTAGCGCTTTATTACAGTACTGGTGGCACTCAGCTTCCA				
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601 CTGGCTCGTCTTTATAATTGATCCAAATGATTCAGAACAGCAAG				
GACCGAGCAAGGAAATTAAACGTAGGTTACTAAAGTCTCGTCGTT				
651 ATTCCAATATATTGAGGGAGAAAATCGCACGAGAATTAGGTACAACCGGA				
TAAGGTTATATAACTCCCTTTACCGTGCTCTTAATCCATGTTGGCCT				
701 GATCTGCACCAGATCTAGCGTAATTACACTTGTGAGAATAGTTGGGGAGA				
CTAGACGTGGCTAGGATCGCATTATGTAACCTTACACCCCTCT				
751 CTTCCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT				
GAAAGGTGACGTTAAGTCTCAGATTGGTCTCGAAACGATCAGGTAA				
801 TCAACTGCAAAGACGTAATGGTCCAATTCACTGAGTGTACGATGTGAGTA				
AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT				
851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCACCA				
ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACCGTGGAGGTGGT				
901 TCGTCACAGTTCTCGTATCTAAAGGCATCGGACGCACCTGATAATGC				
AGCAGTGTCAAAGAACATAGATTCCGTAGCCTGCGTGGACTATTACG				

86/254

FIGURE 17D (CONT'D)

951 TGATGTTGTATGGATCCTGAGCCCATACTGCGTATCGTAGGTCGAAATG
 ACTACAAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC

 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACTACATCCCTACCTCTAAGGTGTTGCTTGCCTTGCCTAT

 1051 CAGTTGTGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACCTT
 GTCAACACCCTGACGTTAGATTATGTCTACGTTAGTCGAGACCTGAAA

 1101 GAAAAGAGACAATACTATTGATCTAATGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC

 1151 GGTACAGTCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATAACACTACTAGATACTAACGTTATGACGACGT

 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGCGACCCTTATACCTTACCTGGTAGTATTAGG

 1251 CAGATCTAGTCTAGTTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTGTCACCATGGTGTG

 1301 TTACAGTCAAACCAACATTTATGCCGTTAGTCAGGTTGGCTTCTACT
 AATGTCACGTTGGTTGTAATACGGCAATCAGTCCAACCGAAGGATGA

 1351 AATAATACACAACCTTTGTTACAACCATGTTGGCTATATGGTCTGTG
 TTATTATGTGTTGAAAACAATGTTGTAACAACCCGATATACCAGACAC

 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCACTGAAA
 GAACGTTCGTTATCACCTGTTACACTATCTCCTGACATCGTCACCTT

 1451 AGGCTGAACAACAGTGGCTCTTATGCAGATGGTCAATACTGCTCAG
 TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

 1501 CAAAAACCGAGATAATTGCCCTACAAGTGATTCTAATATACGGAAACAGT
 GTTTGGCTCTATTACGGAAATGTTCACTAAGATTATATGCCCTTGTCA

 1551 TGTTAAGATCCTCTTGTGGCCCTGCATCCTCTGGCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

 1601 TCAAGAATGATGGAACCATTAAATTGTATAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTTGGTAAAATTAAACATATCACCTAACCAACATCTA

 1651 GTGAGGCATCGGATCCGAGCCTTAAACAAATCATTCTTACCCCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGAGAGGT

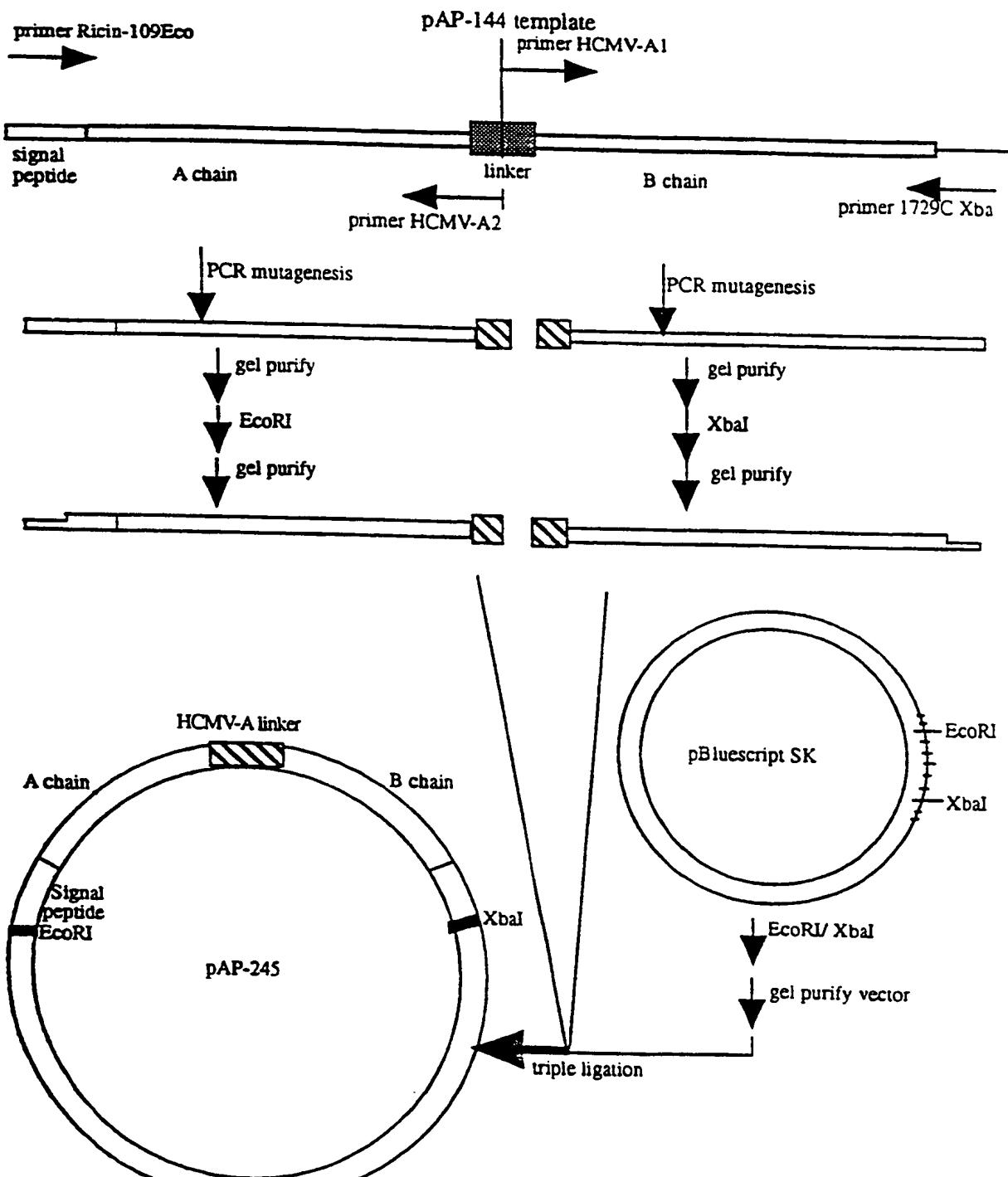
 1701 TGGTGACCCAAACAAATATGGTACCATTTATTTGATAGACAGATTACT
 ACCACTGGGTTGGTTATACCAATGTAATAAAACTATCTGTCTAATGA

 1751 CTCTTGCACTGTTGTCCTGCCATGAAAATAGATGGCTAAATAAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATT

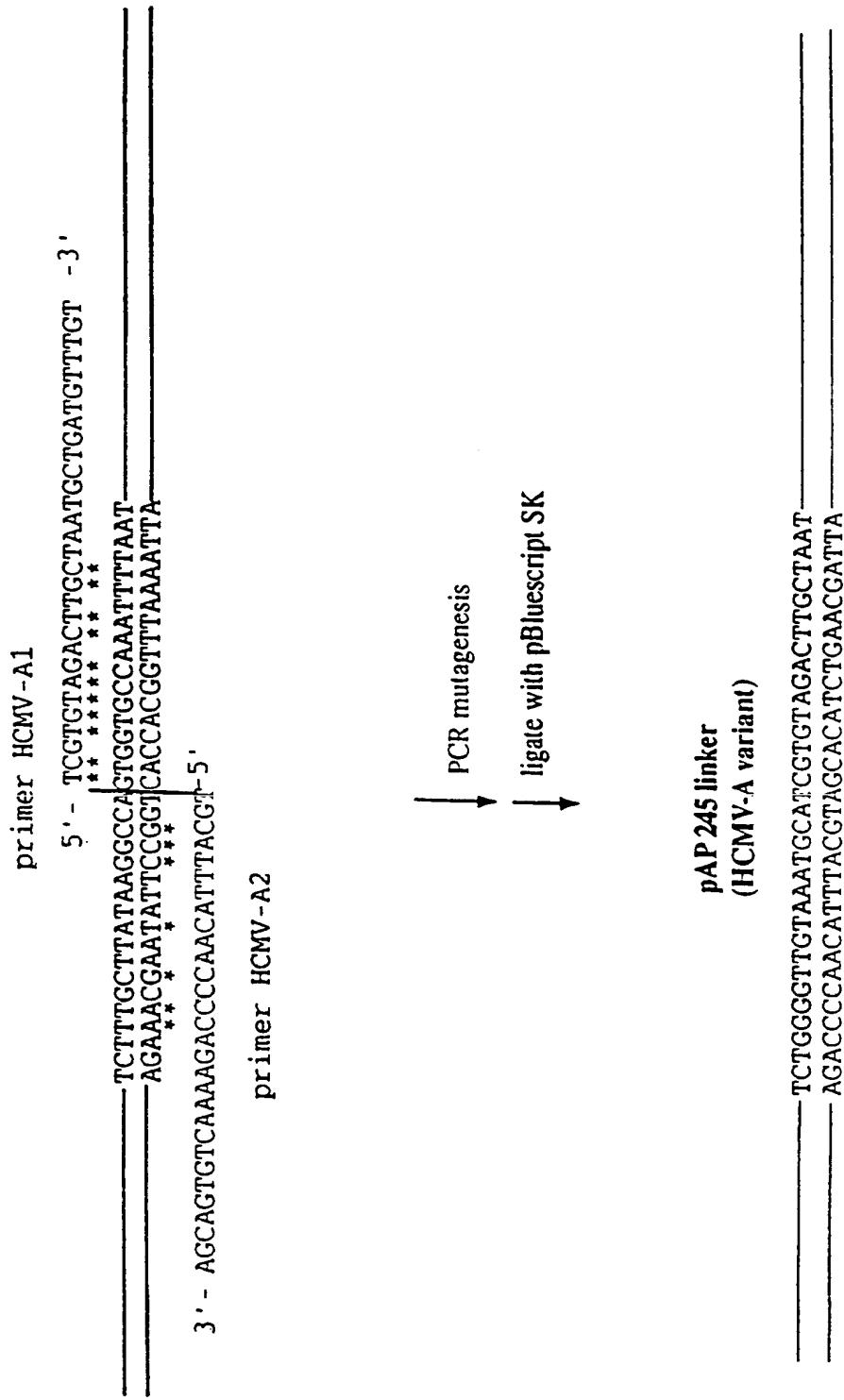
 1801 GGACATTGTAATTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTTAAACATTGACTTTCTGTCGTTCAATATAGCTTAAGG

 1851 TGCAG
 ACGTC

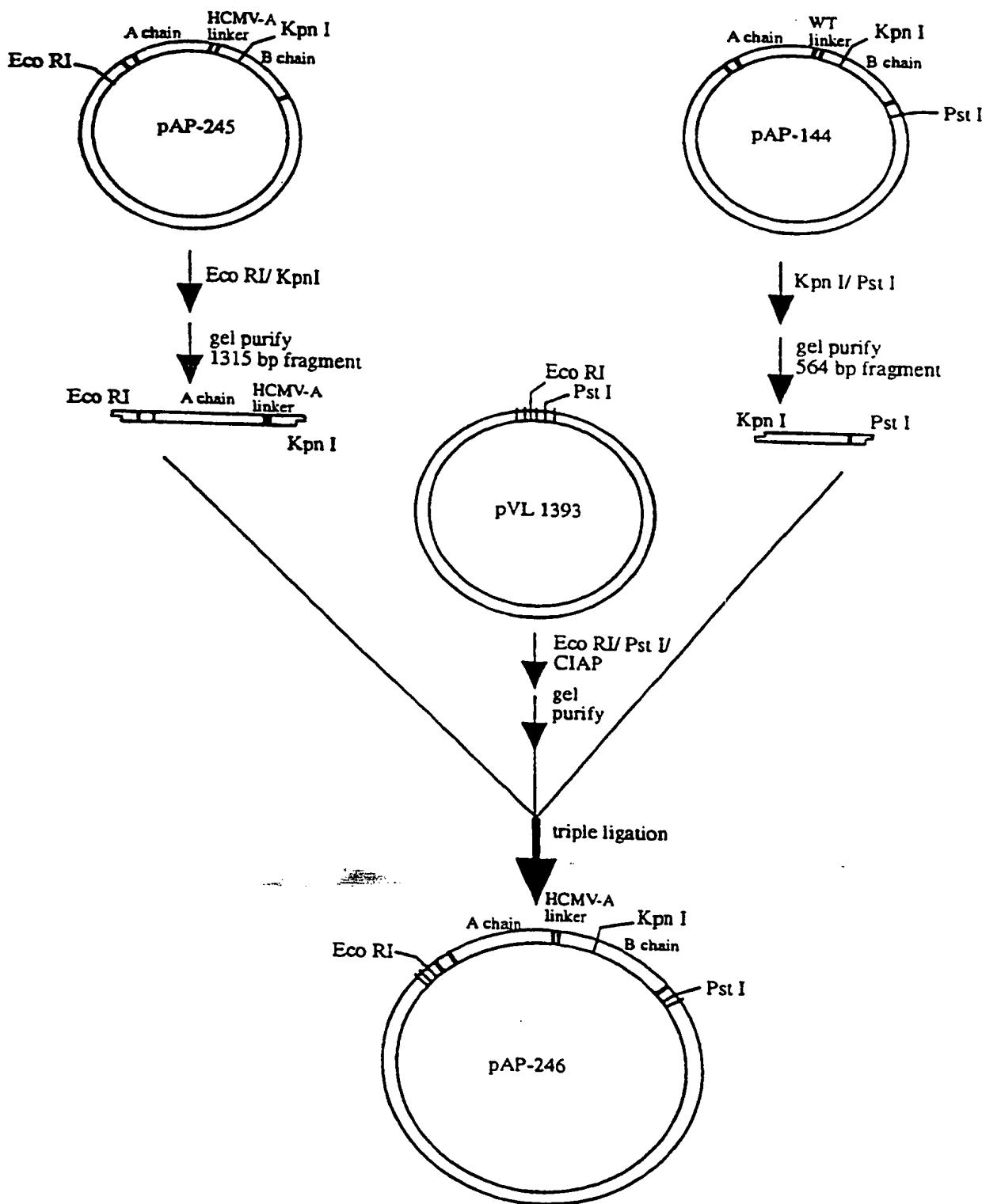
87/254

FIGURE 18A**SUBSTITUTE SHEET (RULE 26)**

88/254

FIGURE 18B**WT preproycin linker**

89/254

FIGURE 18C

90/254

FIGURE 18D

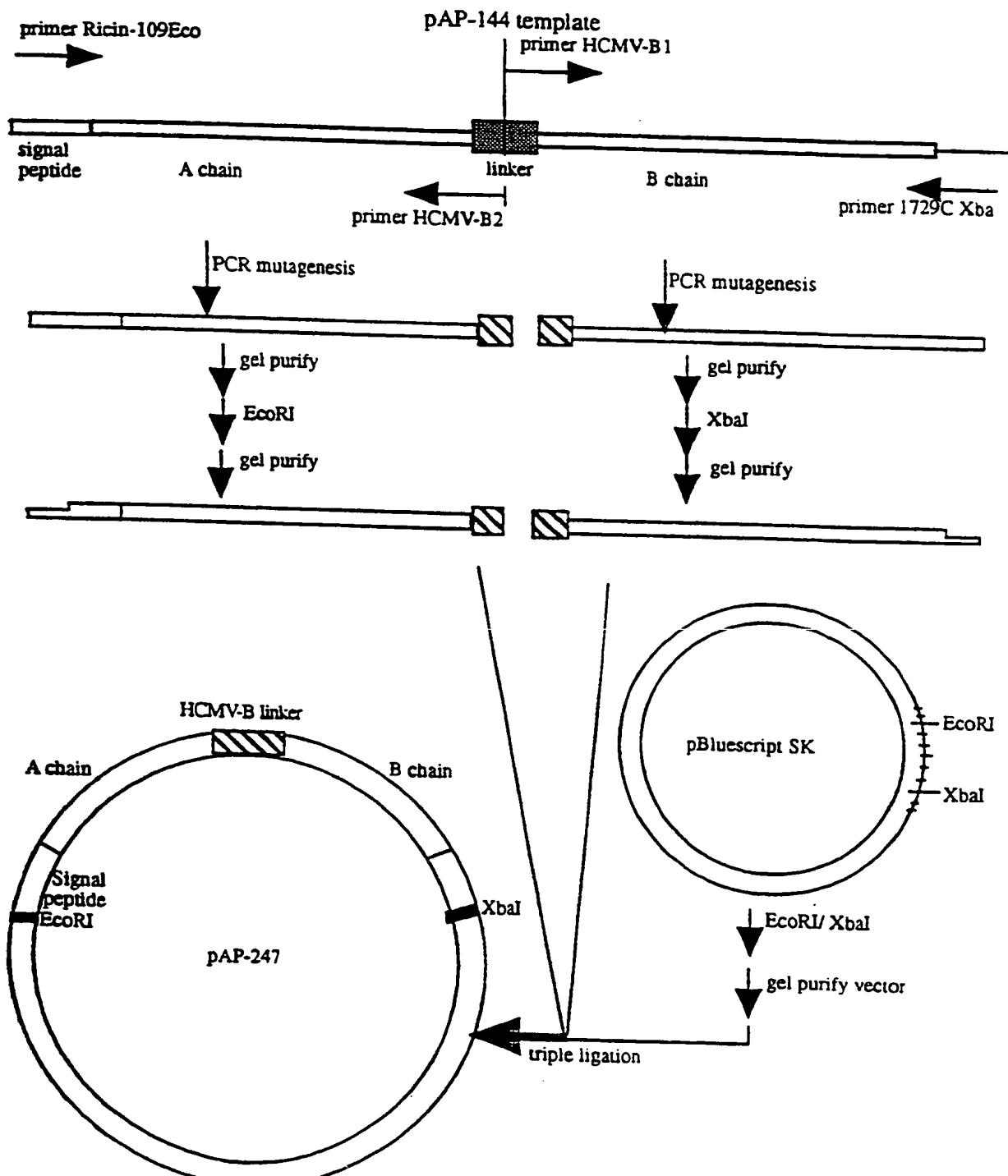
10	20	30	40	50
1 GAATT CATGAAACCGGGAGGAAATACTATTGTAAATGGATGTATGCAGT				
CTTAAGTACTTTGCCCTCTTATGATAACATTACACATACACGTCA				
51 GGCAACATGGCTTGGATCCACCTCAGGGTGGCTTCACATTAG				
CCGTTGTACCGAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC				
101 AGGATAACAACATATTCCCCAAACAATACCAATTATAAACTTTACACAC				
TCCTATTGTTGATAAGGGTTGTTATGGTTAATATTGAAATGGTGT				
151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTGTTGCCGG				
CGCCCCACGGTGACACGTTGATGTGTTGAAATAGTCTCGACAAGCGCC				
201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAAGTGTGCCAA				
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT				
251 ACAGAGTTGGTTGCCTATAAACCAACGGTTTATTTAGTTGAACCTCTCA				
TGTCTAACCAACGGATAATTGGTGCCTAACAAATCAACTTGAGAGT				
301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
TTAGTACGTCTCGAAAGACAAATGTAATCGCGACCTACAGTGGTTACGTAT				
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTCTTCATCCTGACA				
ACACCAGCCGATGGCACGACCTTATCGGTATAAGAAAGTAGGACTGT				
401 ATCAGGAAGATGCAGAACAACTCACTCATCTTCACTGATGTTCAAAAT				
TAGTCCTCTACGTCTCGTTAGTGGAGTAGAAAAGTGAACACTACAAGTTTA				
451 CGATATACATTGCCCTTGGTGGTAATTATGATAGACTTGAACAACTTGC				
GCTATATGTAACCGGAAACCACCAATTAAACTATCTGAACCTTGTGAACG				
501 TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG				
ACCATTAGACTCTCTTATAGCTAACCTTACCAAGGTGATCTCCTCC				
551 CTATCTAGCGCTTATTATTACAGTACTGGTGGCACTCAGCTTCAACT				
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGACTCGAAGGTTGA				
601 CTGGCTCGTCTTATAATTGCACTCAAATGATTTCAGAACAGCAAG				
GACCGAGCAAGGAAATATTAACGTAGGTTACTAAAGTCTCGTCGTT				
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
TAAGGTTATATAACTCCCTCTTACCGGTGCTTTAATCCATGTTGGCCT				
701 GATCTGCACCAAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA				
CTAGACGTGGTCTAGGATCGCATTATGTGAACCTTATCAACCCCCCTCT				
751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT				
GAAAGGTGACGTTAAGTTCTCAGATTGGTCTCGGAAACGATCAGGTAA				
801 TCAACTGCAAAGACGTAATGGTCAAATTCACTGAGTGTACGATGTGAGTA				
AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT				
851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA				
ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACCGGTGGAGGTGGT				
901 TCGTCACAGTTCTGGGGTTGTAATGCATCGTGTAGACTTGCTAATGC				
AGCAGTGTCAAAAGACCCCAACATTACGTAGCACATCTGAACGATTACG				

91/254

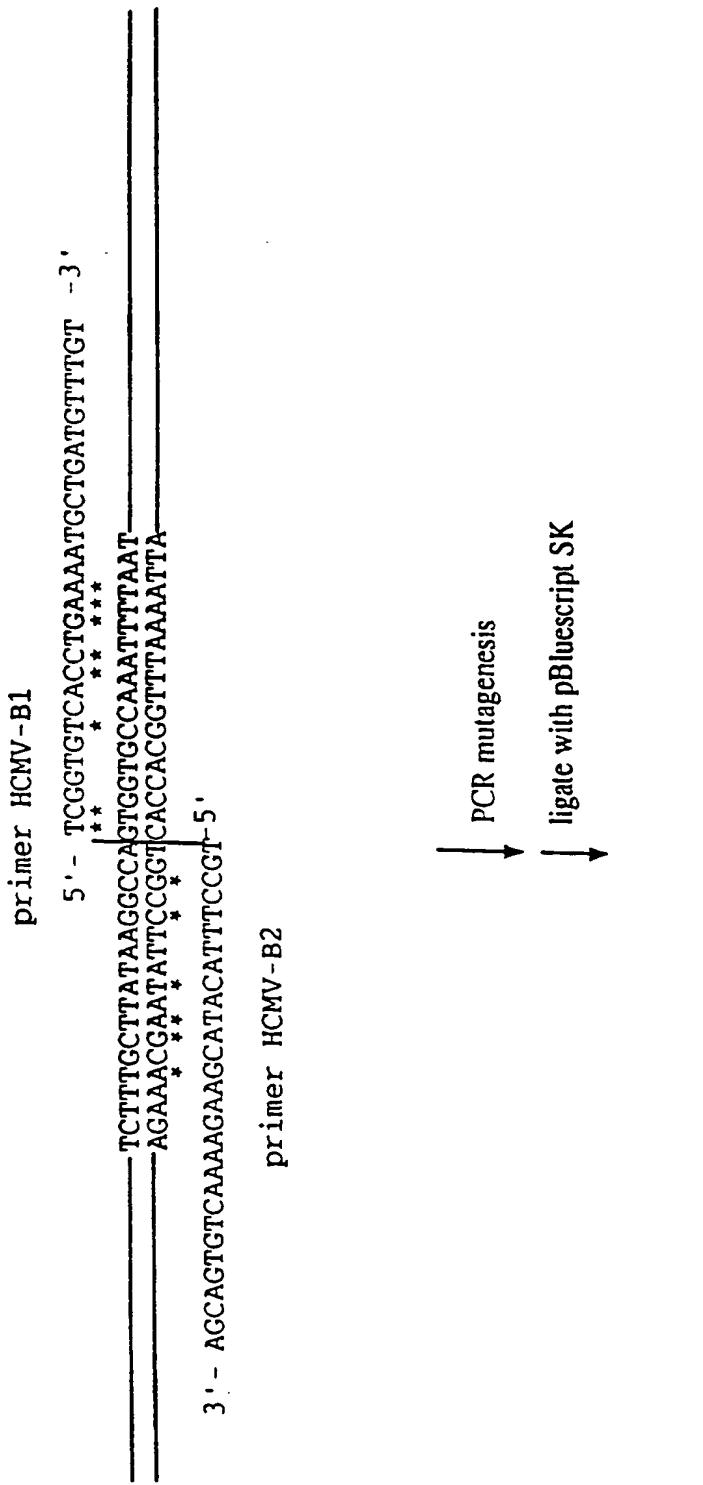
FIGURE 18D (CONT'D)

951 TGATGTTGTATGGATCCTGAGCCCATACTGCCTATCGTAGGTCGAAATG
 ACTACAAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAAACGGAAACGCAATA
 CAGATACACAACATACTACCTACCTCTAAGGTGTTGCCCTTGCCTTAC
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTAGATTATGTCAGCTACGTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAATGGAAGTGTTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
 1151 GGTACAGTCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCACATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCTATTACCTGGTAGTATTAGG
 1251 CAGATCTAGTCTAGTTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTGTACCATGGTGTG
 1301 TTACAGTGCACCAACACATTATGCCGTTAGTCAGGGTTGGCTTCAACT
 AATGTCACGTTGGTTGTAACACGGCAATCAGTCCAAACCGAAGGATGA
 1351 AATAATACACAACCTTTGTTACAACCAATTGTTGGCTATATGGCTGTG
 TTATTATGTTGGAAAACAATGTTGGTAACAACCCGATATACCGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTCGTTATCACCTGTTACACCTATCTCCTGACATCGTCACCTT
 1451 AGGCTGAACAAACAGTGGCTTTATGCAGATGGTCAATACGTCCCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAACCGAGATAATTGCCCTACAAGTGTATTCTAATATACGGAAACAGT
 GTTTGGCTCTATTACGGAATGTTCACTAAGATTATATGCCCTTGTCA
 1551 TGTTAAGATCCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCAATTAAATTGTATAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTGGTAAACATATCACCTAACCAATCTA
 1651 GTGAGGCAGTCGGATCCGAGCCTAAACAAATCATTCTTACCCCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGAACCAACAAATATGGTACCAATTGGTATAGACAGAGATTACT
 ACCACTGGGTTGGTTATACCAATGGTAATAAAACTATCTGTCTAATGA
 1751 CTCTTGCAGTGTGTGTCCTGCCATGAAAATAGATGGCTAAATAAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTGTT
 1801 GGACATTGTAATTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAACATTGACTTCCGTGCTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

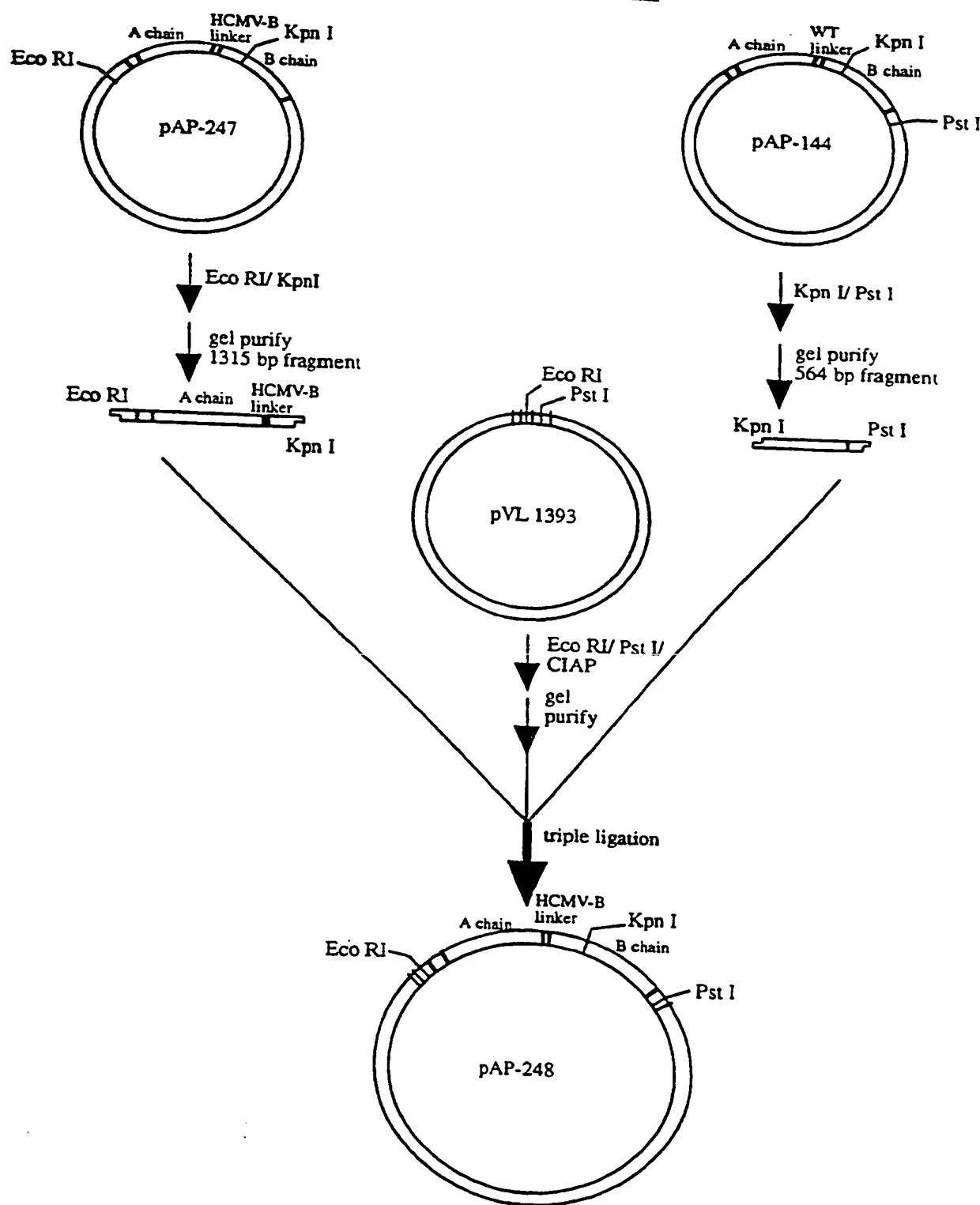
92/254

FIGURE 19A

93/254

FIGURE 19B**WT preprotein linker****pAP247 linker
(HCMV-B variant)**

94/254

FIGURE 19C

SUBSTITUTE SHEET (RULE 26)

95/254

FIGURE 19D

10 20 30 40 50

```

1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGCCCTCTTATGATAACATTATACTACATACTACGTCA

51  GGCAACATGGCTTGGATCCACCTCAGGGTGGCTTCACATTAG
   CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAAGTGTAACTC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCA
   TCCTATTGTTGTATAAGGGGTTGTTATGGGTTAATATTTGAAATGGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTGTTCGCGG
   CGCCCACGGTGACACGTTCGATGTGTTGAAATAGTCTCGACAAGCGCC

201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATAACCAGTGTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACCTCTCA
   TGTCCTAACCAACGGATATTGGTTGCCAAATAAACTCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGTGGAAATAGCGCATATTCTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTATCGGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCCTCATCTTCACTGATGTTAAAAAT
   TAGCCTTCTACGTCTCGTTAGTGAAGAAAAGTGAACACAAGTTTA

451 CGATATACATTGCCCTTGGTGGTAATTATGATAGACTTGAACAACCTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTTTATAGCTAACCCCTTACCGGTGATCTCCTCC

551 CTATCTCAGCGCTTATTATTACAGTACTGGTGGCACTCAGCTTCAACT
   GATAGAGTCGCGAAATAATAATGTCATGACCAACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCTTATAATTGATCCAAATGATTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAACCGTAGGTTACTAAAGTCTCGTGTTC

651 ATTCATATATTGAGGGAGAAATGCGCAGGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTTTACCGTGTCTTAATCCATGTTGGCCT

701 GATCTGCACCAAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA
   CTAGACGTGGTAGGATCGCATTATGTGAACCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT
   GAAAGGTGACGTTAAGTCTCAGATTGGTCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTCAAATTCACTGATGTCAGATGAGTA
   AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGATAGATGCGCACCTCCACCA
   ATAATTAGGGATAGTATCGAGAGTACCCACATATCTACCGGTGGAGGTGGT

901 TCGTCACAGTTCTCGTATGAAAGGCATCGGTGTACCTGAAAATGC
   AGCAGTGTCAAAAGAACATACATTCCGTAGCCACAGTGGACTTTACG

```

96/254

FIGURE 19D (CONT'D)

951 TGATGTTGTATGGATCCTGAGCCCATACTGCGTATCGTAGGTCGAAATG
 ACTACAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC

 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACTACATCCCTACCTCTAAGGTGTTGCCCTTGCCTTAT

 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTAGATTATGTCAGTTAGTCGAGACCTGAAA

 1101 GAAAAGAGACAATACTATTGATCTAATGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC

 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATAACACTAGATACTAACGTTATGACGACGT

 1201 ACTGATGCCACCCGCTGGCAAATATGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGCGACCCTTATACCTTACCTGGTAGTATTAGG

 1251 CAGATCTAGTCTAGTTTAGCAGCGACATCAGGGAACAGTGGTACACAC
 GTCTAGATCAGATCAAATCGCTGCTAGTCCCTGTCACCATGGTGTG

 1301 TTACAGTCAAACCAACATTATGCCGTTAGTCAAGGTTGGCTTCCTACT
 AATGTCACGTTGGTTGTAACCGCAATCAGTCCAACCGAAGGATGA

 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTTGGAAACAAATGTTGTAACAAACCGATATACCAACAGACAC

 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGA
 AACGTTCTTATCACCTGTTACACCTATCTCCTGACATCGTCACTTT

 1451 AGGCTGAACAAACAGTGGCTCTTATGCAGATGGTCAATACGTCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

 1501 CAAAACCGAGATAATTGCCCTTACAAGTGATTCTAATATACGGAAACAGT
 GTTTGGCTCTATTAAACGGAAATGTTACTAAGATTATATGCCCTTGTC

 1551 TGTTAAGATCCTCTTGTGGCCCTGCATCCTCTGGCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

 1601 TCAAGAAATGATGGAACCATTAAATTGTTAGTGGATTGGTTAGAT
 AGTTCTTACTACCTTGGTAAATTAACATATCACCTAACCAACAACTCTA

 1651 GTGAGGCATGGATCCGAGCCTTAAACAAATCATTCTTACCCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT

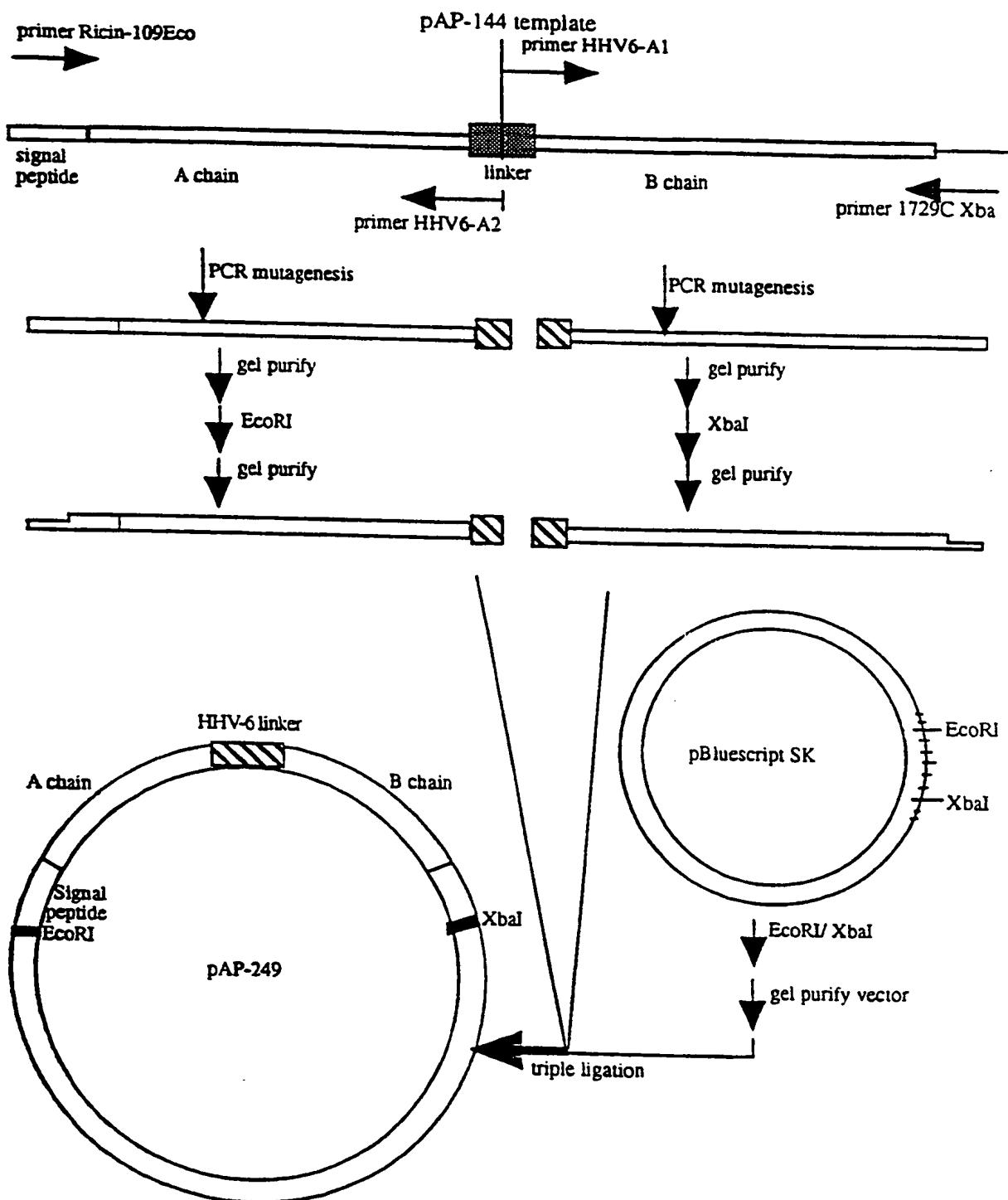
 1701 TGGTGACCCAAACAAATATGGTACCTTATTGATAGACAGATTACT
 ACCACTGGGTTGGTTATACCAATGTAATAAAACTATCTGTCTAATGA

 1751 CTCTTGCACTGTTGTCCTGCCATGAAAATAGATGGCTAAATAAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATT

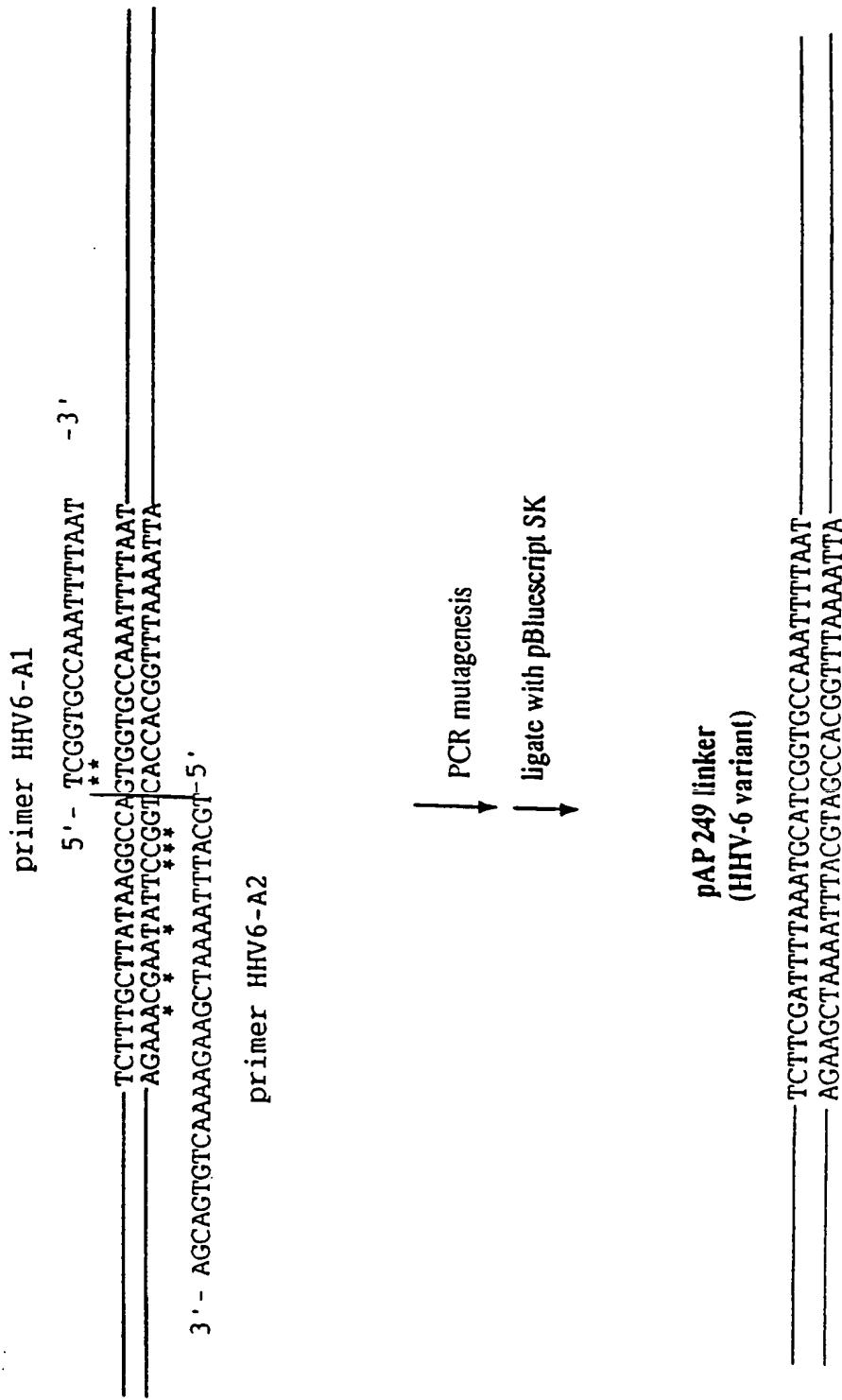
 1801 GGACATTGTAATTGTAACGTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAACATTGACTTTCTGTCGTTCAATATAGCTTAAGG

 1851 TGCAG
 ACGTC

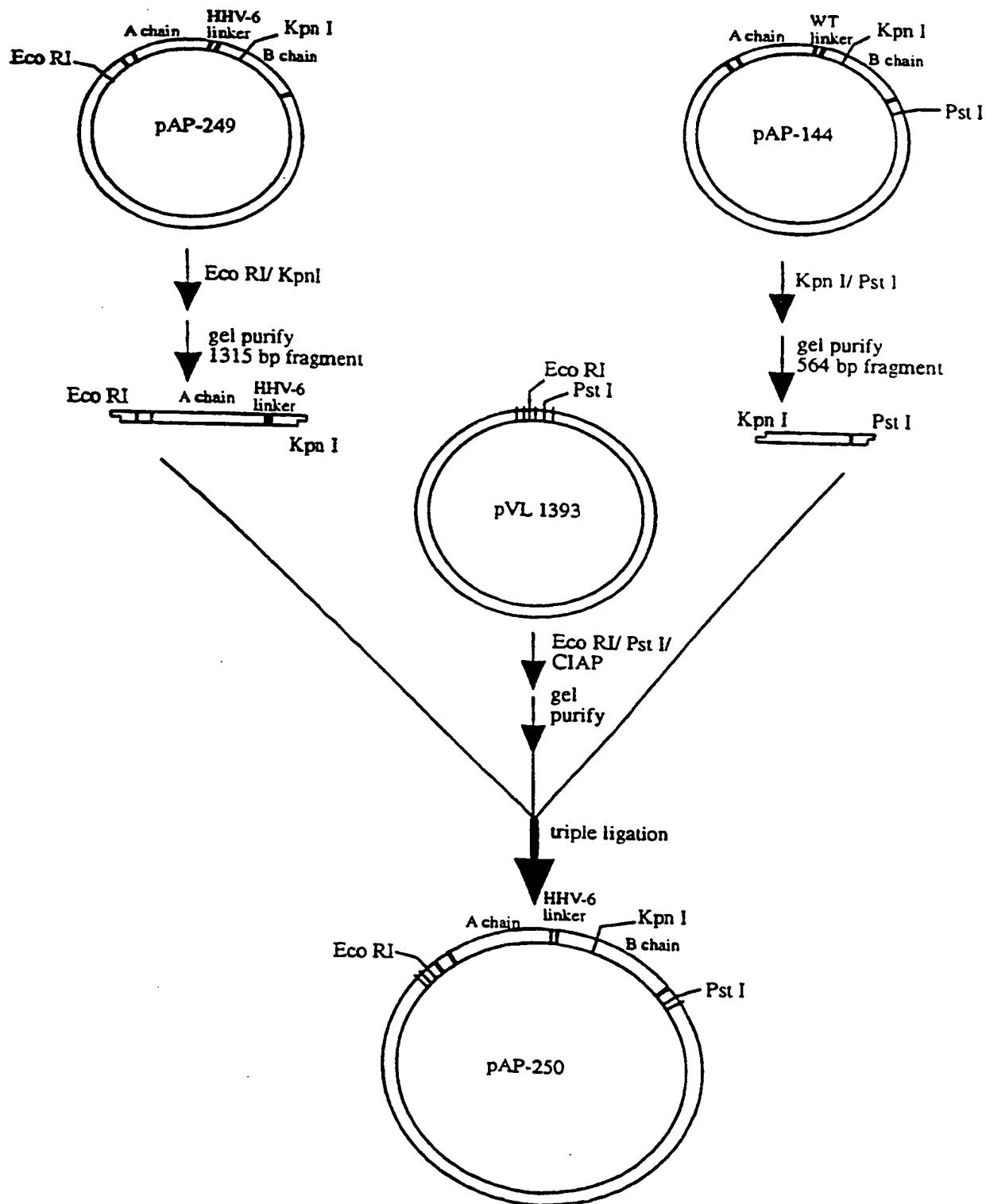
97/254

FIGURE 20A

98/254

FIGURE 20B**WT preprorocin linker**

99/254

FIGURE 20C

SUBSTITUTE SHEET (RULE 26)

100/254

FIGURE 20D

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
 CTTAAGTACTTGGCCCTCTTATGATAACATTATACCTACATACGTCA
 51 GGCAACATGGCTTGTTGGATCCACCTCAGGGTGGCTTTCACATTAG
 CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTATC
 101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTACCA
 TCCTATTGTTGTATAAGGGGTTGTTATGGGTTAATATTGAAATGGTGT
 151 GCGGGTGCCACTGTGCAAAGCTACACAAAACCTTTATCAGAGCTGTTGCC
 CGCCCCACGGTGACACGTTCGATGTGTTGAAATAGTCTCGACAAGCGCC
 201 TCGTTAACAAACTGGAGCTGATGTGAGACATGATATAACCAGTGTGCCAA
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGTT
 251 ACAGAGTTGGTTGCCTATAAACCAACGGTTTTAGTTAGTTGAACCTCTCA
 TGTCTCAACCAAACGGATATTGGTTGCCAAATAAAACTAAGTGTGAGAGT
 301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
 351 TGTGGTCGGCTACCGTGTGGAAATAGCGCATATTCTTCATCCTGACA
 ACACCAGCCGATGGCACGACCTTATCGGTATAAGAAAGTAGGACTGTT
 401 ATCAGGAAGATGAGCAACTCACTCATCTTCACTGATGTTCAAAAT
 TAGTCCTCTACGTCTCGTTAGTGAGTAGAAAGTGAACATCAAGTTTA
 451 CGATATACTCGCCTTGGGTGTAATTATGATAGACTTGAACAACTTGC
 GCTATATGTAAGGGAAACCAACCATTAATACTATCTGAACCTGTTGAACG
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG
 ACCATTAGACTCTTTATAGCTCAACCCCTTACCAAGGTGATCTCCTCC
 551 CTATCTCGCGCTTATTACAGTACTGGTGGCACTCAGCTTCAACT
 GATAGAGTCGCGAAATAATAATGTATGACCAACCGTGAGTCGAAGGTTGA
 601 CTGGCTCGTCTTATAATTGATCCAAATGATTTGAGCAAGCAGCAAG
 GACCGAGCAAGGAAATATTAACGTTAGGTTACTAAAGTCTCGTGTTC
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
 TAAGGTATATAACTCCCTTTACGCGTGTCTTAATCCATGTTGGCCT
 701 GATCTGCACCAAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA
 CTAGACGTGGTCTAGGATCGCATTAAATGTGAACCTTATCAACCCCTCT
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT
 GAAAGGTGACGTTAAGTTCTCAGATTGGTCTCGAACGATCAGGTTA
 801 TCAACTGCAAAGACGTAATGGTCCAATTCACTGAGTGTACGATGTGAGTA
 AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACGCGTGGAGGTGGT
 901 TCGTCACAGTTCTCGATTAAATGCATCGGTGCCAAATTAAATGCA
 AGCAGTGTCAAAAGAAGCTAAATACGTAGCCACGGTTAAAATTACG

101/254

FIGURE 20D (CONT'D)

951 TGATGTTGTATGGATCCTGAGCCCATAGTCGTATCGTAGGTCGAAATG
 ACTACAAACATACTACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC

 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACTACCTACCTCTAAGGTGTTGCCTTGCGTTAT

 1051 CAGTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTAGATTATGTCAGTTAGTCGAGACCTGAAA

 1101 GAAAAGAGACAATACTATTGATCTAATGGAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC

 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTAGATACTAACGTTATGACGACGT

 1201 ACTGATGCCACCGCTGGCAAATATGGGATAATGGAACCACATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCTTACCTTGTAGTATTAGG

 1251 CAGATCTAGTCTAGTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCTTGTCACCATGGTGTG

 1301 TTACAGTGCAAACCAACATTATGCCGTTAGTCAGGTTGGCTTCCTACT
 AATGTCACGTTGGTTGTAACAGGCAATCAGTTCAACCGAAGGATGA

 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTTGGAAAACAATGTTGTAACAAACCGATATACCAACAC

 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTGTTATCACCTGTCACCTATCTCCTGACATCGTCACCTT

 1451 AGGCTGAACAAACAGTGGCTTTATGCAGATGGTCAATACGTCCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

 1501 CAAACCGAGATAATTGCCCTACAAGTGATTCTAATATACGGGAAACAGT
 GTTTGGCTCTATTACGGAATGTTCACTAAGATTATATGCCCTTGTCA

 1551 TGTTAAGATCCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

 1601 TCAAGAATGATGGAACCATTAAATTGTTAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTGGTAAATTAAACATATCACCTAACCAATCTA

 1651 GTGAGGCAGTCGGATCCGAGCCTAACAAATCATTCTTACCCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT

 1701 TGGTGAACCAAACAAATATGGTTACCATTATTTGATAGACAGATTACT
 ACCACTGGGTTGGTTATACCAATGTAATAAAACTATCTGTCTAATGA

 1751 CTCTTGCAAGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
 GAGAACGTACACACACAGGACGGTACTTTATCTACCGAATTATTTT

 1801 GGACATTGTAATTGGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAAACATTGACTTCCCTGTCGTTCAATATAGCTTAAGG

 1851 TGCAG
 ACGTC

102/254

FIGURE 21

Ricin linker (wild type) :

A chain- S L L I R P V V P N F N -B chain

pAP-213/pAP-214 linker (Cathepsin B) :

A chain- S L L K S R M V P N F N -B chain

pAP-215/pAP-216 linker (MMP-3) :

A chain- R P K P Q Q F F G L M N -B chain

pAP-217/pAP-218 linker (MMP-7) :

A chain- S L R P L A L W R S F N -B chain

pAP-219/pAP-220 linker (MMP-9) :

A chain- S P Q G I A G Q R N F N -B chain

pAP-221/pAP-222 linker (THERMOLYSIN-LIKE MMP) :

A chain- D V D E R D V R G F A S F L -B chain

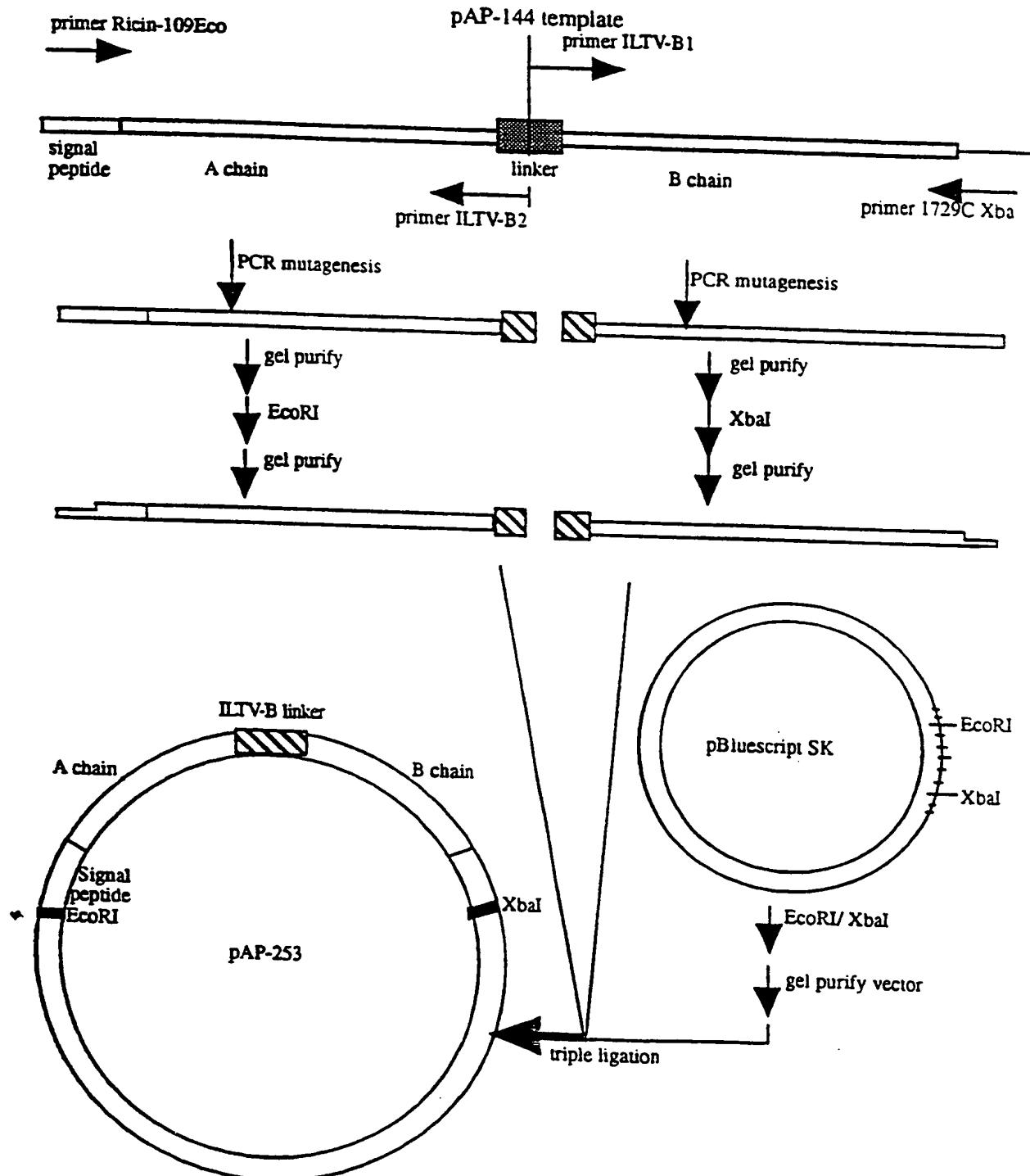
pAP-241/pAP-242 linker (EBV-A) :

A chain- S K L V Q A S A S G V N -B chain

pAP-243/pAP-244 linker (EBV-B) :

A chain- S S Y L K A S D A P D N -B chain

103/254

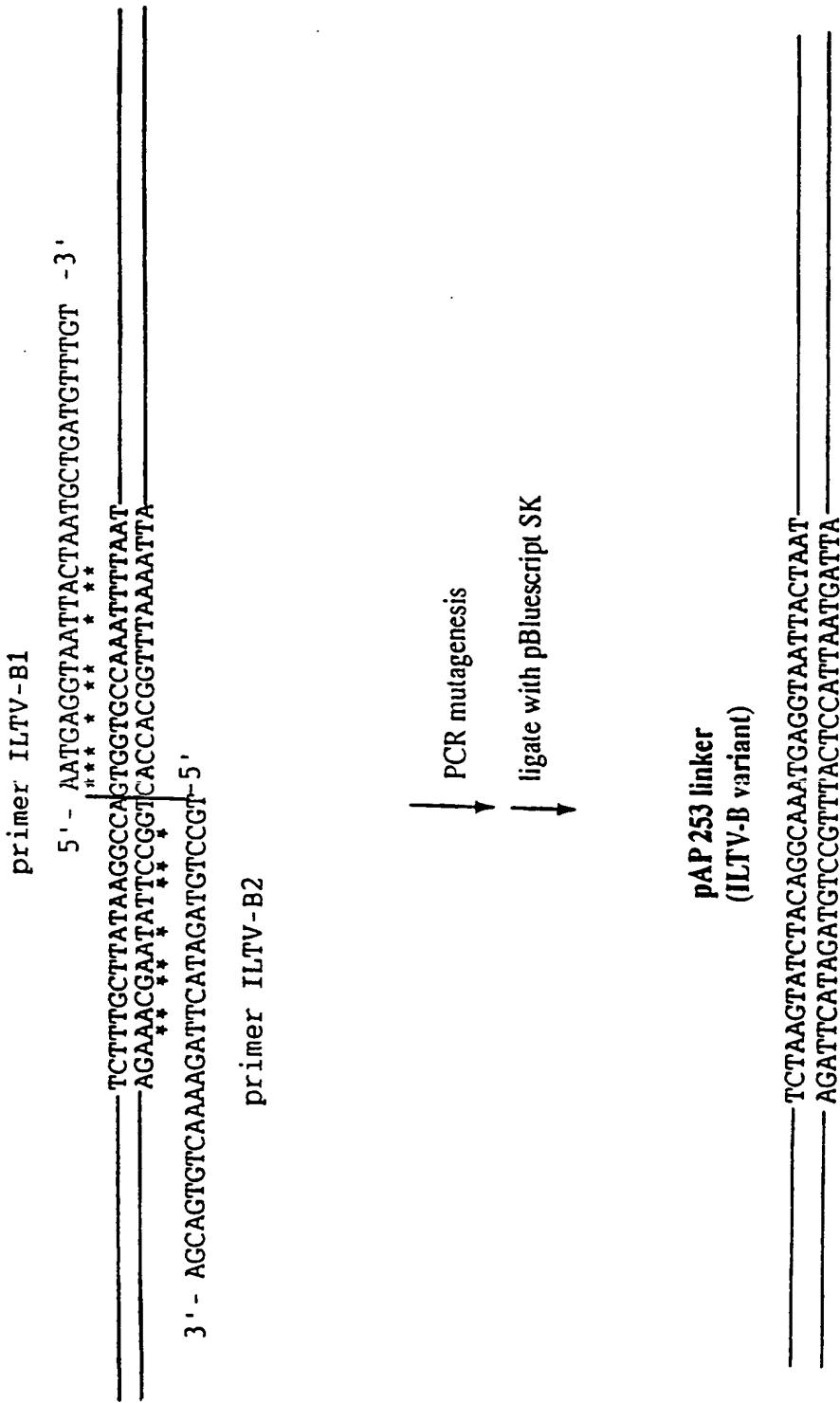
FIGURE 22A

SUBSTITUTE SHEET (RULE 26)

104/254

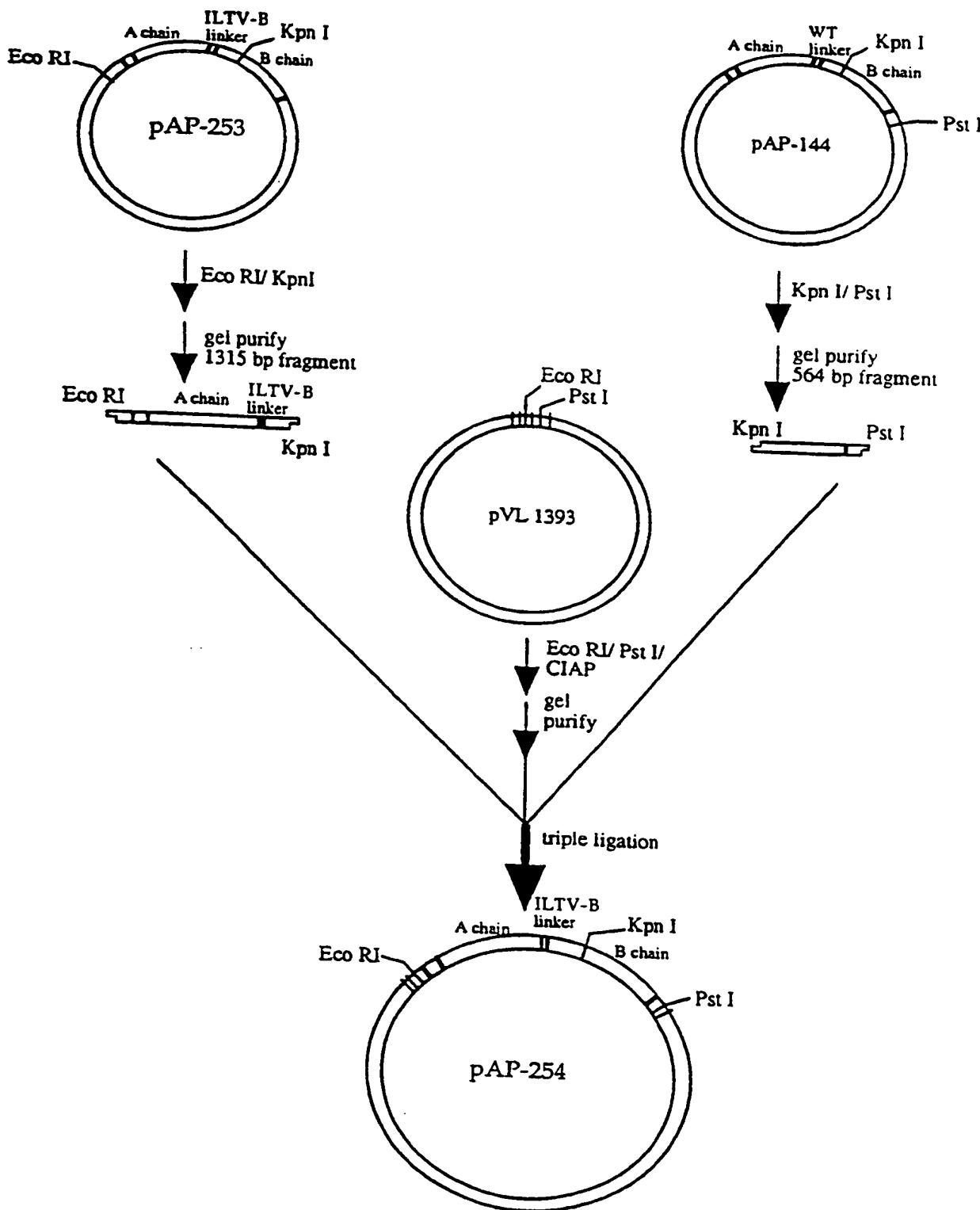
FIGURE 22B

WT preprorocin linker



SUBSTITUTE SHEET (RULE 26)

105/254

FIGURE 22C

106/254

FIGURE 22D

10 20 30 40 50

```

1  GAATT CATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGCCCTCCTTATGATAACATTACACATACGTCA
51  GGCAACATGGCTTGTTGGATCCACCTCAGGGTGGTCTTCACATTAG
   CCGTTGTACCGAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAA
101 AGGATAACAAACATATTCCCCAAACAATACCCAATTATAAACTTTACCA
   TCCTATTGTTGATAAGGGTTTGTATGGTTAATATTGAAATGGTGT
151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTGTTCGCGG
   CGCCCACGGTGACACGTTCGATGTGTTGAAATAGTCTCGACAAGCGCC
201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAAGTGTGCCAA
   AGCAAATTGTTGACCTCGACTACACTGTACTATATGGTCACAACGGTT
251 ACAGAGTTGGTTGCCCTATAAAACCAACGGTTATTTAGTTGAACCTCTCA
   TGTCTCACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACTACAGTGGTTACGTAT
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTATCGGTATAAAGAAAGTAGGACTGT
401 ATCAGGAAGATGCGAGAACAACTCACTCATCTTCACTGATGTTCAAAAT
   TAGTCCTTCTACGTCTCGTTAGTGAGTAGAAAGTGAACACTACAGTTTA
451 CGATATACATTGCCCTTGGTGGTAATTATGATAGACTTGAACAACTTGC
   GCTATATGTAAGCGGAAACACCATTAAACTATCTGAACCTTGTGAACG
501 TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTTTTATAGCTCACCCCTTACCGAGTGATCTCCTCC
551 CTATCTCAGCGCTTTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   GATAGAGTCGCGAAATAATAATGTCACTGACCACCGTGAGTCGAAGGTTGA
601 CTGGCTCGTTCTTATAATTGCACTCCAAATGATTTCAAGCAGCAAG
   GACCGAGCAAGGAAATATTAACGTAAGGTTACTAAAGTCTCGTGTTC
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTTTACCGTGCTCTTAACTCCATGTTGGCCT
701 GATCTGCACCAAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA
   CTAGACGTGGTCTAGGATCGCATTAAATGTGAACCTTATCAACCCCTCT
751 CTTTCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT
   GAAAGGTGACGTTAAGTTCTCAGATTGGTCTCGGAAACGATCAGGTTA
801 TCAACTGCAAAGACGTAATGGTCCAAATTCACTGAGTGTACGATGAGTA
   AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT
851 TATTAATCCCTATCATAGCTCTCATGGTGATAGATGGCACCTCCACCA
   ATAATTAGGGATAGTATCGAGAGTACCCACATCTACCGTGGAGGTGGT
901 TCGTCACAGTTCTAAGTATCTACAGGCAAATGAGGTAATTACTAATGC
   AGCAGTGTCAAAGATTCAAGATGTCCTTACTCCATTAAATGATTACG

```

107/254

FIGURE 22D (CONT'D)

951 TGATGTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGCAAATG
 ACTACAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGAAACGCAATA
 CAGATACACAACATACTACCCCTACCTCTAAGGTGTTGCCCTTGCCTTAT

 1051 CAGTTGTGGCCATGCAAGTCTAATAACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTAGTCTACGTTAGTCAGACCTGAAA

 1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC

 1151 GGTACAGTCCGGAGTCTATGTGATGATCTATGATTGAAACTGCTGCA
 CCATGTCAGGCCCTCAGATAACACTAGATAACTAACGTTATGACGACGT

 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAATCC
 TGACTACGGTGGCGACCGTTATACCTTACCTGGTAGTATTAGG

 1251 CAGATCTAGTCTAGTTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCTTGTCACCATGGTGTG

 1301 TTACAGTGCAAACCAACATTATGCCGTTAGTCAGGTTGGCTTCCACT
 AATGTCACGTTGGTTGTAACAGGCAATCAGTCCAACCGAAGGATGA

 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTTGGAAAACAATGTTGTAACAACCCGATATACAGACAC

 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTAAA
 GAACGTTGTTATCACCTGTTACCTATCTCCTGACATCGTCACCTT

 1451 AGGCTGAACAAACAGTGGCTCTTATGCGAGATGGTCAATACGTCCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCACCAAGTTATGCAAGGAGTC

 1501 CAAAACCGAGATAATTGCCCTACAAGTGATTCTAATATACGGGAAACAGT
 GTTTGGCTCTATTAACGGAATGTTACTAAGATTATATGCCCTTGTCA

 1551 TGTTAAGATCCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCCGGACGTAGGAGACCGGTTGCTACCTACA

 1601 TCAAGAATGATGGAACCATTAAATTGTATAGTGGATTGGTGTAGAT
 AGTTCTTACCTGGTAAATTAACATATCACCTAACCAATCTA

 1651 GTGAGGCAGTCGGATCCGAGCCTTAAACAAATCATTCTTACCCCTCTCCA
 CACTCCGCTAGGCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT

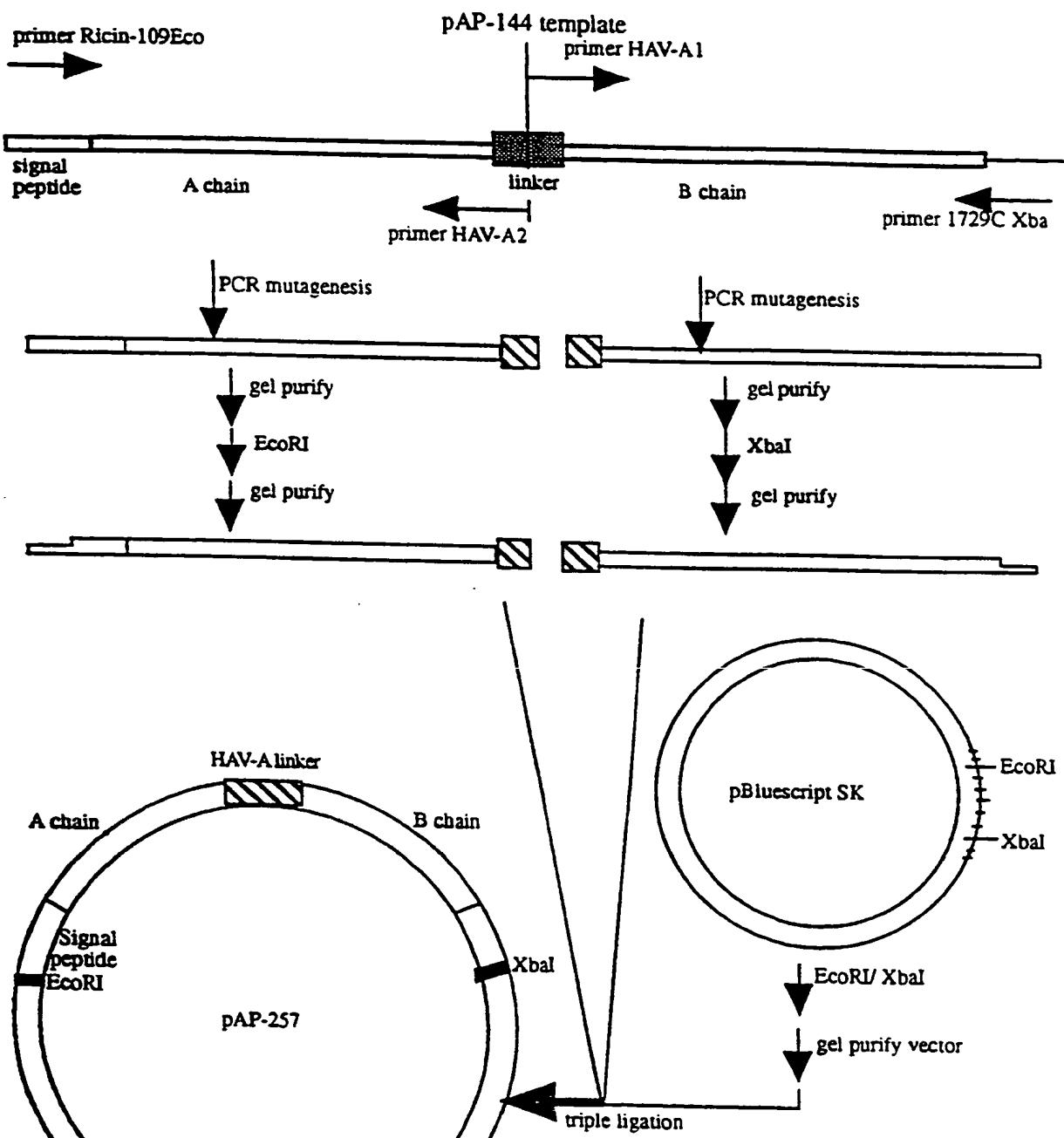
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTGATAGACAGATTACT
 ACCACTGGGTTGGTTATACCAATGGAATAAAACTATCTGTCTAATGA

 1751 CTCTTGAGTGTTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTT

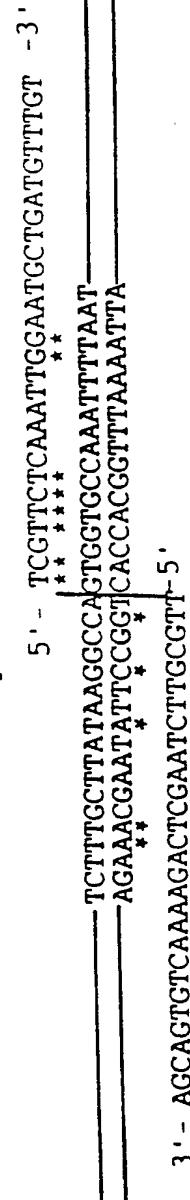
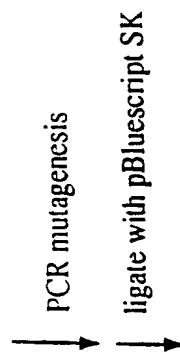
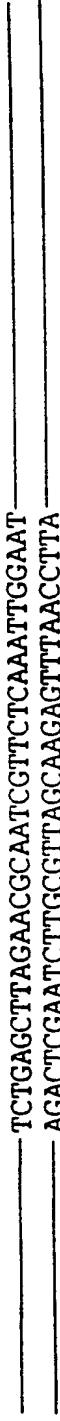
 1801 GGACATTGTAATTGTAACGTAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

 1851 TGCAG
 ACGTC

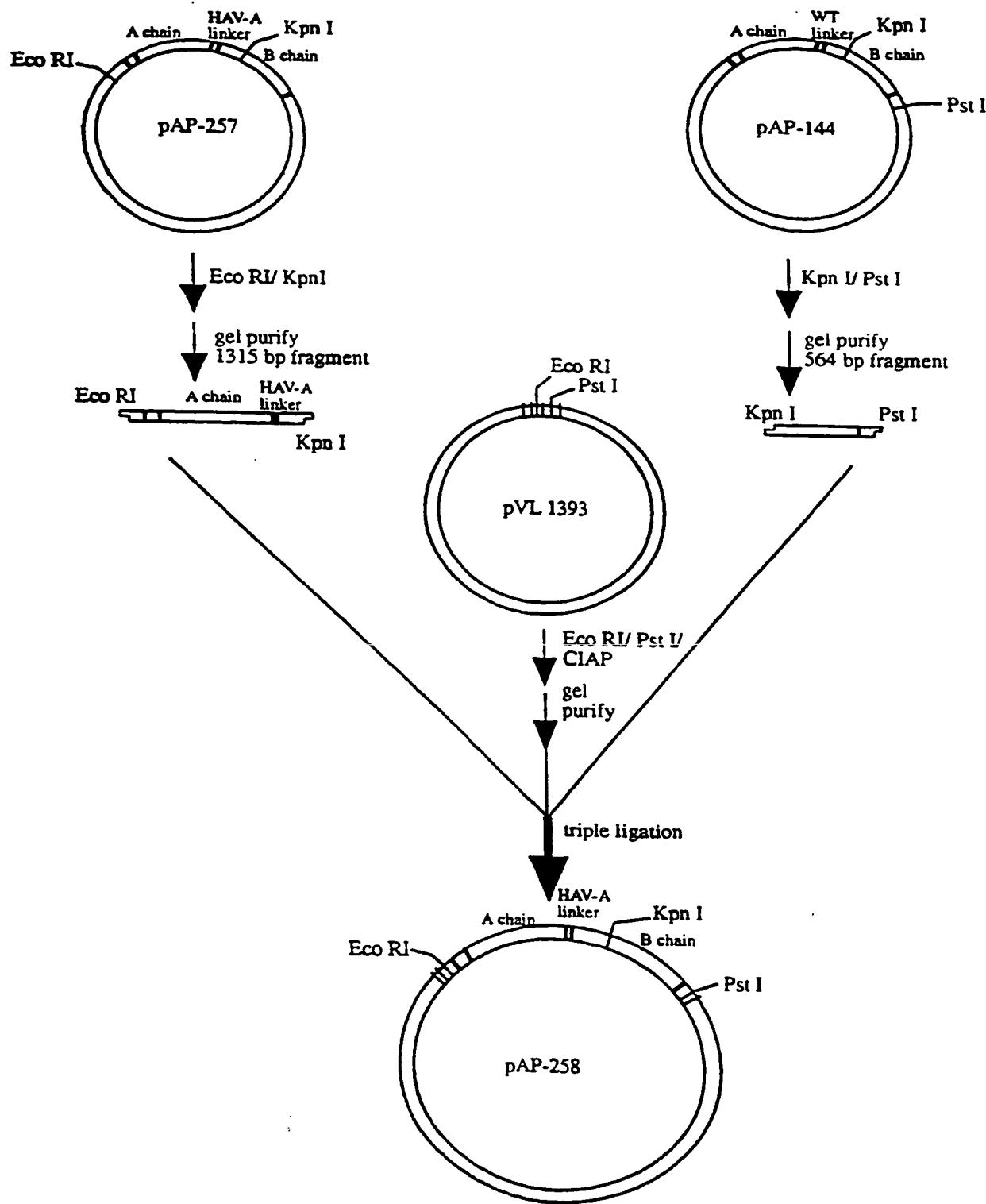
108/254

FIGURE 23A

109/254

FIGURE 23B**WT preprorcin linker****primer HAV-A1****primer HAV-A2****pAP257 linker
(HAV-A variant)**

110/254

FIGURE 23C**SUBSTITUTE SHEET (RULE 26)**

111/254

FIGURE 23D

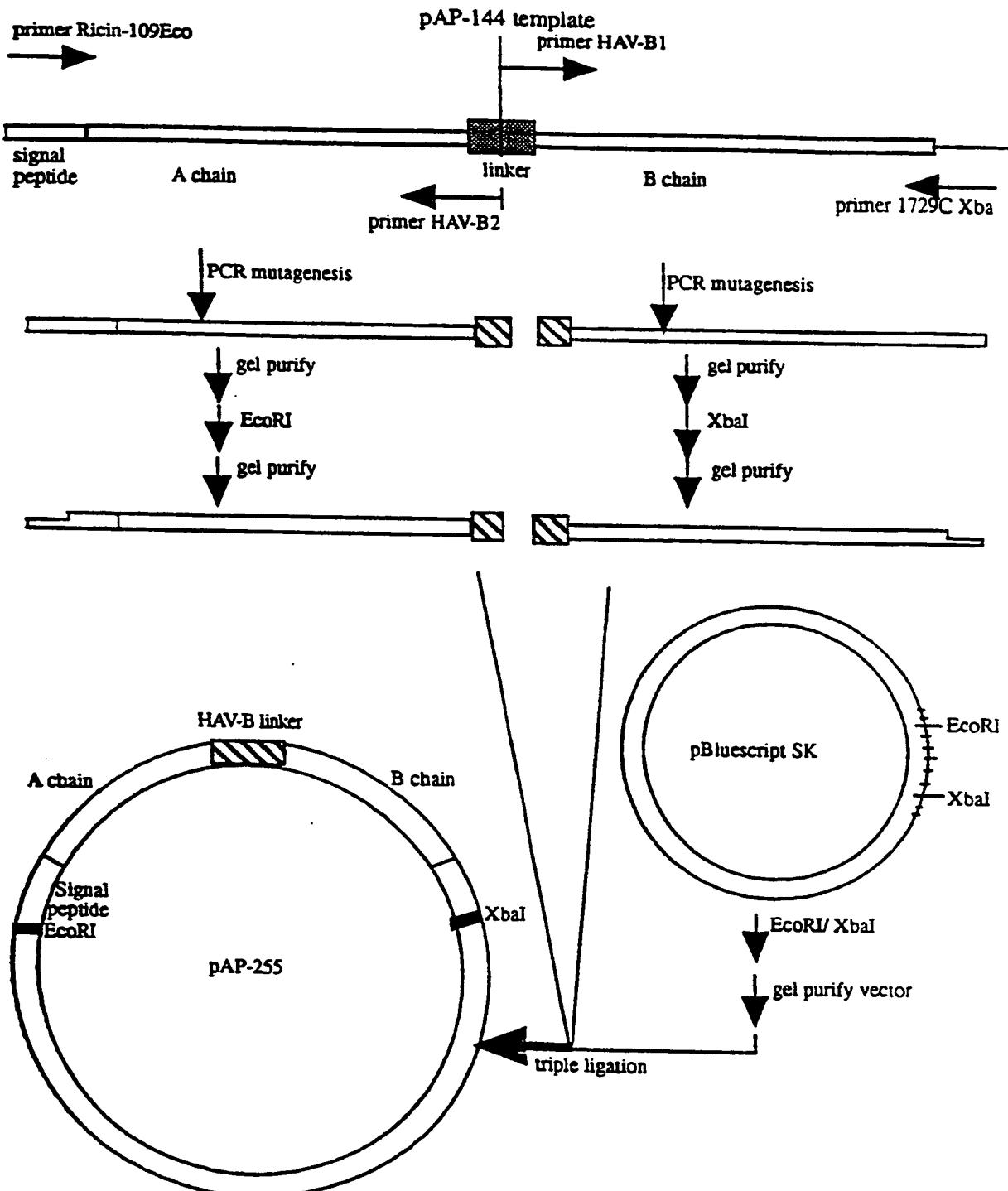
10	20	30	40	50
1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTATGATAACATTACCTACATACGTCA			
51 GGCAACATGGCTTGTTGGATCCACCTCAGGGTGGTCTTCACATTAG				
	CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAA			
101 AGGATAAACACATATTCCCCAACAAACCAATTATAAATTTACCAACA				
	TCCTATTTGTGTATAAGGGTTTGTATGGTTAATATTGAAATGGTGT			
151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTGTTGCCG				
	CGCCCCACGGTGACACGTTGATGTGTTGAAATAGTCTCGACAAGGCC			
201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAAGTGTGCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGACTATATGGTCACAACGGTT			
251 ACAGAGTTGGTTGCCCTATAAACCAACGGTTATTTAGTTGAACCTCA				
	TGTCTCAACCAACGGATAATTGGTTGCCAAATAAAACTCAACTTGAGAGT			
301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCACCTACAGTGGTTACGTAT			
351 TGTGGTCGGCTACCGTGTGGAAATAGCGCATATTCTTCATCCTGACA				
	ACACCAGCCGATGGCACGACCTTATCGCGTATAAAAGAAAGTAGGACTGT			
401 ATCAGGAAGATGCAGAACAACTCATCTTTCACTGATGTTCAAAT				
	TAGTCCTCTACGTCTCGTTAGTGAAGAAAAGTGAACACTAACAGTTTA			
451 CGATATACATTGCCCTTGGTGGTAATTATGATAGACTTGAACAACTTGC				
	GCTATATGTAAGCGGAAACCACCATTAATAACTATCTGAACCTGTTGAACG			
501 TGGTAATCTGAGAGAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTTTTATAGCTAACCCTTACCGAGTGTATCCTCC			
551 CTATCTAGCGCTTTATTACAGTACTGGTGGCACTCAGCTTCAACT				
	GATAGAGTCGCGAAATAATAATGTCATGACCAACCGTGAAGTTGA			
601 CTGGCTCGTCTTTATAATTGACATCCAAATGATTCAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTGTTC			
651 ATTCCAATATATTGAGGGAGAAATGCCACGGAAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTTTACGGTGTCTTAATCCATGTTGGCCT			
701 GATCTGACCAAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA				
	CTAGACGTGGTCTAGGATCGATTAATGTAACCTTATCAACCCCCCTCT			
751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCAAAT				
	GAAAGGTGACGTTAAGTCTCAGATTGGTCTCGGAAACGATCAGGTTA			
801 TCAACTGCAAAGACGTAATGGTCCAATTCACTGAGTGTACGATGTGAGTA				
	AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT			
851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCCACCTCCACCA				
	ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACGGTGGAGGTGGT			
901 TCGTCACAGTTTCTGAGCTTAGAACGCAATCGTTCTCAAATTGGAATGC				
	AGCAGTGTCAAAGACTCGAACCTTACGCGTTAGCAAGAGTTAACCTTACG			

112/254

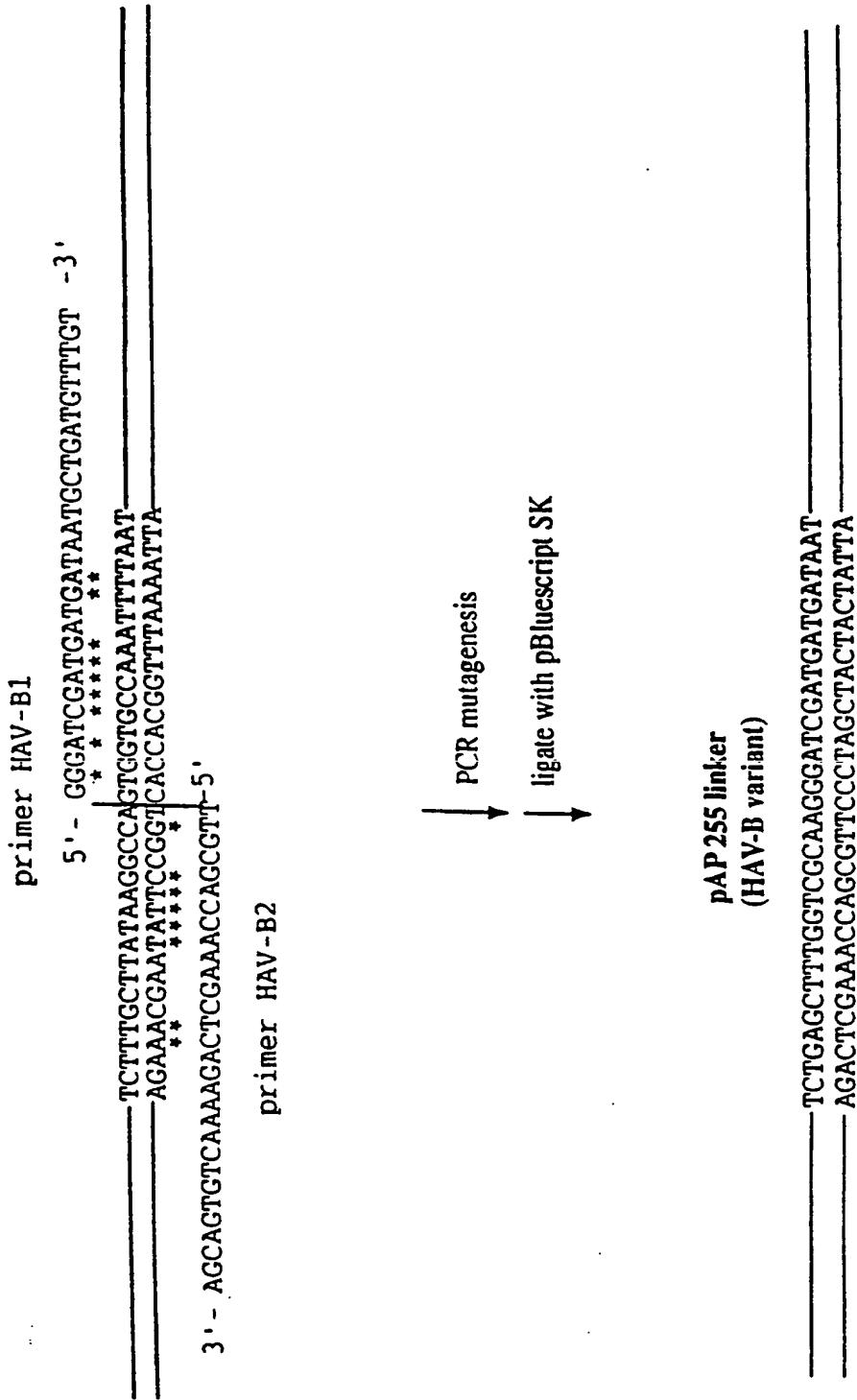
FIGURE 23D (CONT'D)

951 TGATGTTGTATGGATCTGAGCCCATAGTCGTATCGTAGGTCGAATG
 ACTACAAACATAACCTAGGACTCGGTATCACGCATAGCATCCAGCTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGAAACGCAATA
 CAGATACACAACATAACATCCCTACCTCTAAGGTGTTGCCCTTGCCTAT
 1051 CAGTTGTGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTAGATTATGCTACGTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGAGTCTATGTGATGATCTATGATTGCAAACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCACATCAAATCC
 TGACTACGGTGGCGACCCTTATACCCATTACCTGGTAGTATTAGG
 1251 CAGATCTAGTCTAGTTTAGCAGCGACATCAGGAAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAATCGCGCTGTAAGTCCCTGTACCATGGTGTG
 1301 TTACAGTGCACCAACATTATGCCGTTAGTCAGGTTGGCTTCTACT
 AATGTCACGTTGGTTGTAATACGGCAATCAGTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGCTGTG
 TTATTATGTGTTGAAAACAATGTTGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGGACTGTAGCAGTGAAA
 GAACGTTCGTTATCACCTGTTACACCTATCTCTGACATCGTCACTTT
 1451 AGGCTGAACAAACAGTGGCTTTATGCAGATGGTCAATACGTCCCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCCTACAAGTGATTCTAATATACGGAAACAGT
 GTTTGGCTCTATTACCGGAATGTTCACTAAGATTATATGCCCTTGTCA
 1551 TGTTAAGATCCTCTTGTCGGCCCTGCATCCTCTGGCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTTAAATTGTTAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTGGTAAATTAACATATCACCTAACCAATCTA
 1651 GTGAGGCAGTCGGATCCGAGCCTTAAACAAATCATTCTTACCCCTCTCCA
 CACTCCGCTAGCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAAATATGGTTACCATTTGATAGACAGATTACT
 ACCACTGGGTTGGTTATACCAATGTAATAAAACTATCTGTCTAATGA
 1751 CTCTTGCACTGTGTGTCCTGCCATGAAAATAGATGGCTAAATAAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTT
 1801 GGACATTGTAATTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAAACATTGACTTTCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

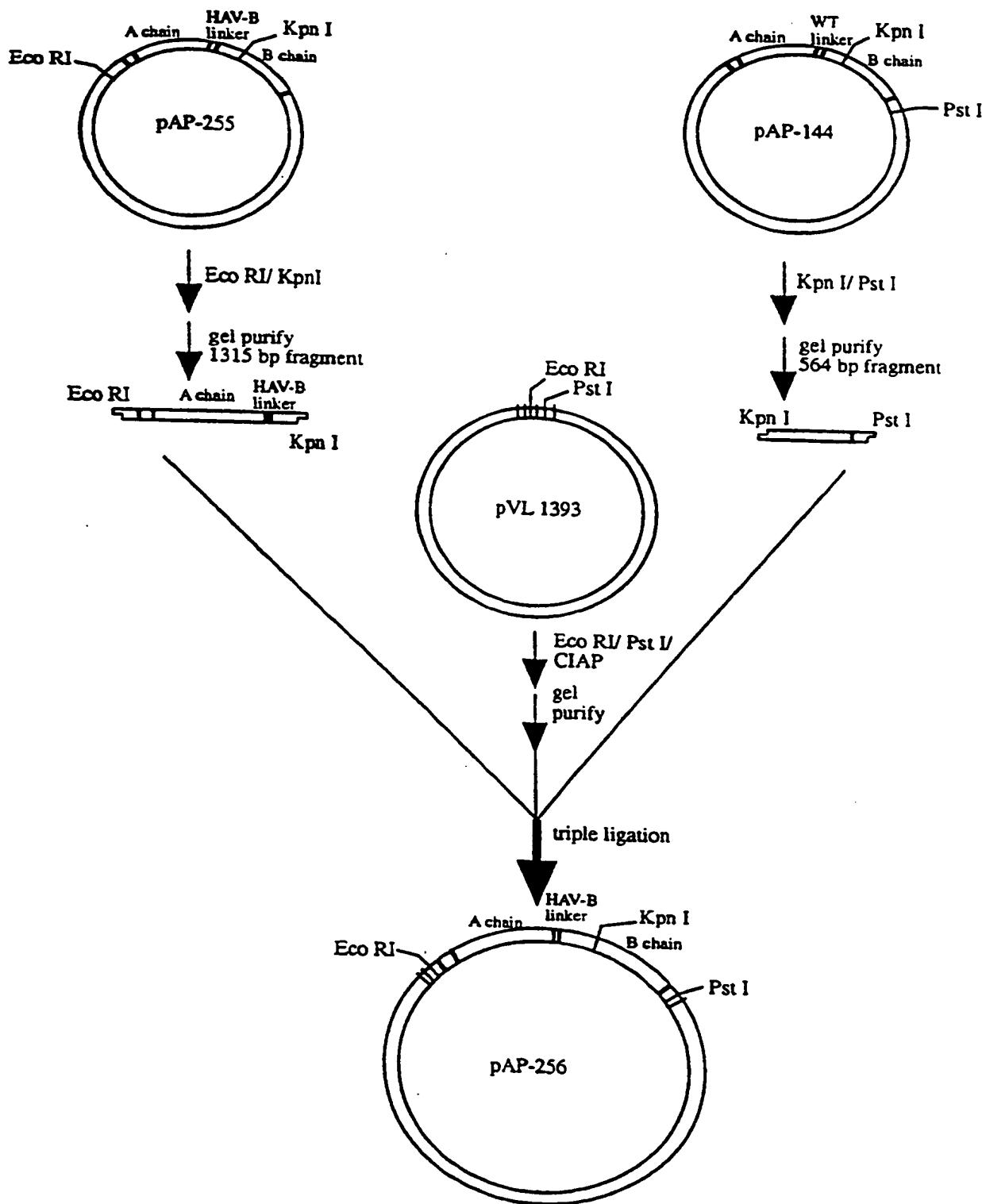
113/254

FIGURE 24A**SUBSTITUTE SHEET (RULE 26)**

114/254

FIGURE 24B**WT preprorocin linker****SUBSTITUTE SHEET (RULE 26)**

115/254

FIGURE 24C

SUBSTITUTE SHEET (RULE 26)

116/254

FIGURE 24D

10	20	30	40	50
1	GAATTCATGAAACGGGAGGAAATACTATTGTAATATGGATGTATGCAGT			
	CTTAAGTACTTTGCCCTCCTTATGATAACATTATACTACATACGTCA			
51	GGCAACATGGCTTGTGATCCACCTCAGGGTGGCTTCACATTAG			
	CCGTTGTACCGAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAA			
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTACCA			
	TCCTATTGTTGTATAAGGGTTGTTATGGGTTAATATTGAAATGGTGT			
151	GCGGGTGCCTACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTCGCG			
	CGCCCACGGTGACACGTTGATGTGTTGAAATAGTCTCGACAAGCGCC			
201	TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAAGTGTGCAA			
	AGCAAATTGTTGACCTCGACTACACTCTGACTATATGGTCACAACGGTT			
251	ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACCTCTCA			
	TGTCTCAACCAACGGATATTGGTTGCCAAATAAACTCAACTTGAGAGT			
301	AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA			
	TTAGTACGTCTCGAAAGACAAATGTAATCGCACCTACAGTGGTTACGTAT			
351	TGTGGTCGGTACCGTGTGGAAATAGCGCATATTTCTTCATCCTGACA			
	ACACCAGCCGATGGCACGACCTTATCGCGTATAAAAGAAAGTAGGACTGT			
401	ATCAGGAAGATGCAGAACACTCATCTTTCACTGATGTTCAAAAT			
	TAGTCCCTCTACGTCTCGTTAGTAGAAAAGTGAACACTACAAGTTTA			
451	CGATATACATTGCCCTTGGTGGTAATTATGATAGACTTGAACAACTTGC			
	GCTATATGTAAGCGGAAACCACCAATTAACTATCTGAACCTGTTGAACG			
501	TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG			
	ACCATTAGACTCTCTTATAGCTCAACCCTTACAGGTGATCTCCTCC			
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCAACT			
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA			
601	CTGGCTCGTTCTTATAATTGATCCAAATGATTCAGAAGCAGCAAG			
	GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTCGTT			
651	ATTCCAATATATTGAGGGAGAAATGCCACGAGAATTAGGTACAACCGGA			
	TAAGGTTATATAACTCCCTCTTACCGCTGCTTAAATCCATGTTGGCCT			
701	GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA			
	CTAGACGTGGTCTAGGATCGCATTAAATGTGAACCTTATCAACCCCTCT			
751	CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCAAAT			
	GAAAGGTGACGTTAAGTCTCAGATTGGTCTCGAAACGATCAGGTTA			
801	TCAACTGCAAAGACGTAATGGTCCAATTCACTGAGTACGATGTGAGTA			
	AGTTGACGTTCTGCATTACCAAGGTTAAAGTCACACATGCTACACTCAT			
851	TATTAATCCCTATCATAGCTCTCATGGTGTATAAGATGCGCACCTCCACCA			
	ATAATTAGGGATAGTATCGAGAGTACACATATCTACCGCTGGAGGTGGT			
901	TCGTCACAGTTCTGAGCTTGGTCGAAGGGATCGATGATGATAATGC			
	AGCAGTGTCAAAGACTCGAAACCAGCGTCCCTAGCTACTACTATTACG			

117/254

FIGURE 24D (CONT'D)

951 TGATGTTGTATGGATCCTGAGCCCAGTAGTCGTACGTAGGTCGAAATG
 ACTACAAACATACTACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC

 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACTACCTACCTTAAGGTGTTGCCTTGCGTTAT

 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTAGATTATGTCACGTTAGTCGAGACCTGAAA

 1101 GAAAAGAGACAATACTATTGATCTAATGGAAGTGTAACTACTTACG
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC

 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCACATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCCATTACCTGGTAGTATTTAGG

 1251 CAGATCTAGTCTAGTTTAGCAGCAGACATCAGGGAACAGTGGTACCCACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCTTGTACCATGGTGTG

 1301 TTACAGTCAAACCAACATTATGCCGTTAGTCAGGTTGGCTTCTACT
 AATGTCACGTTGGTTAAATACGCAATCAGTCCAACCGAAGGATGA

 1351 AATAATACACAAACCTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG
 TTATTATGTTGGAAAACAATGTTGGTAACAACCGATATACCAGACAC

 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAA
 GAACGTTCGTTATCACCTGTTACACCTATCTCCTGACATCGTCACTTT

 1451 AGGCTGAACAAACAGTGGCTCTTATGCAGATGGTCAATACGTCCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

 1501 CAAAACCGAGATAATTGCTTACAAGTGATTCTAATATAACGGAAACAGT
 GTTTGGCTCTATTACGGAATGTTACTAAGATTATGCCCCTTGTCA

 1551 TGTTAAGATCCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

 1601 TCAAGAATGATGGAACCATTAAATTGTATAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTTGGTAAATTAAACATATCACCTAACCAACAACTCTA

 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTACCCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGAGAGGT

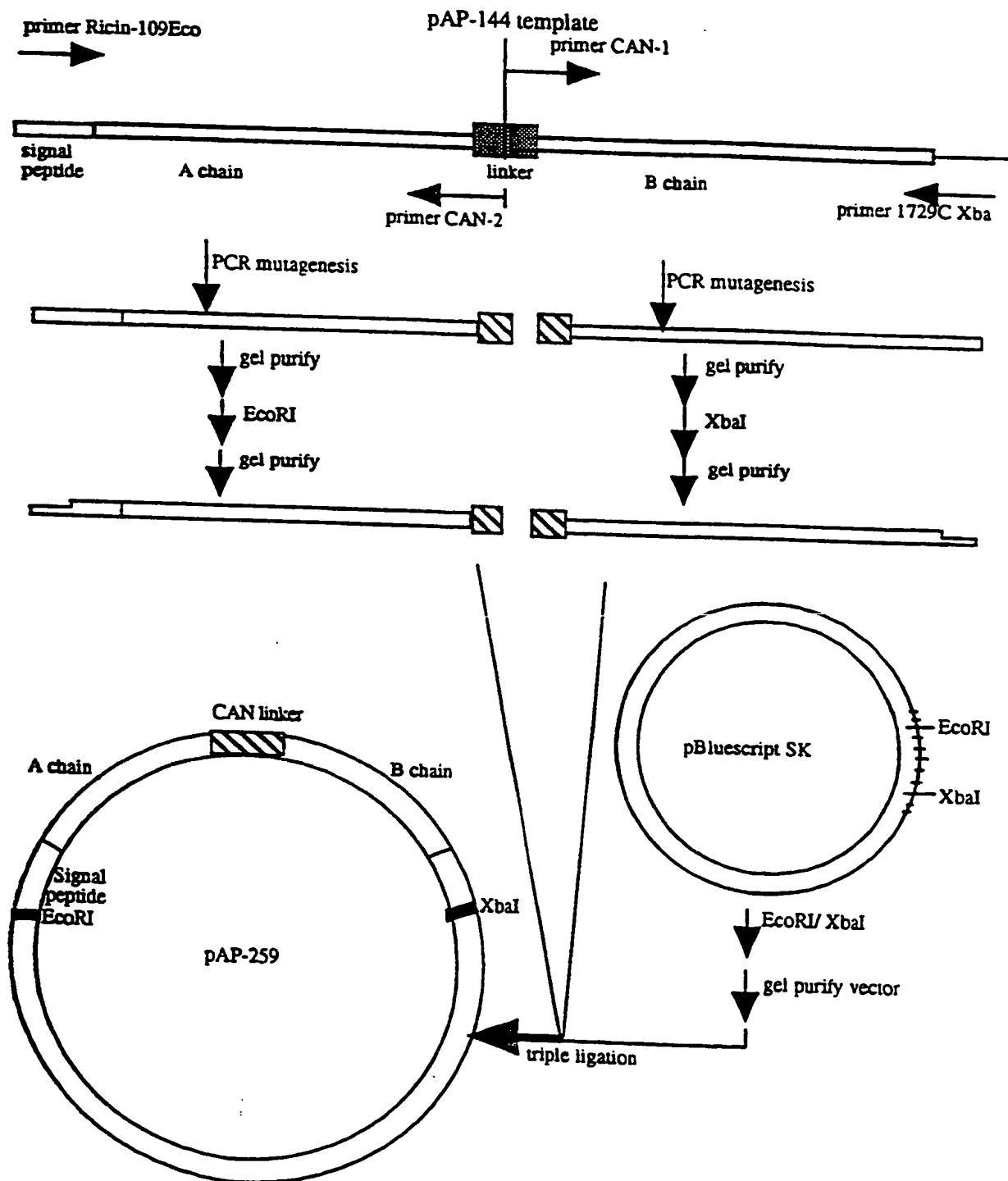
 1701 TGGTGACCCAAACCAAATATGGTACCAATTATTGATAGACAGATTACT
 ACCACTGGGTTGGTTATACCAATGGAATAAAACTATCTGTCTAATGA

 1751 CTCTGCAGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTTT

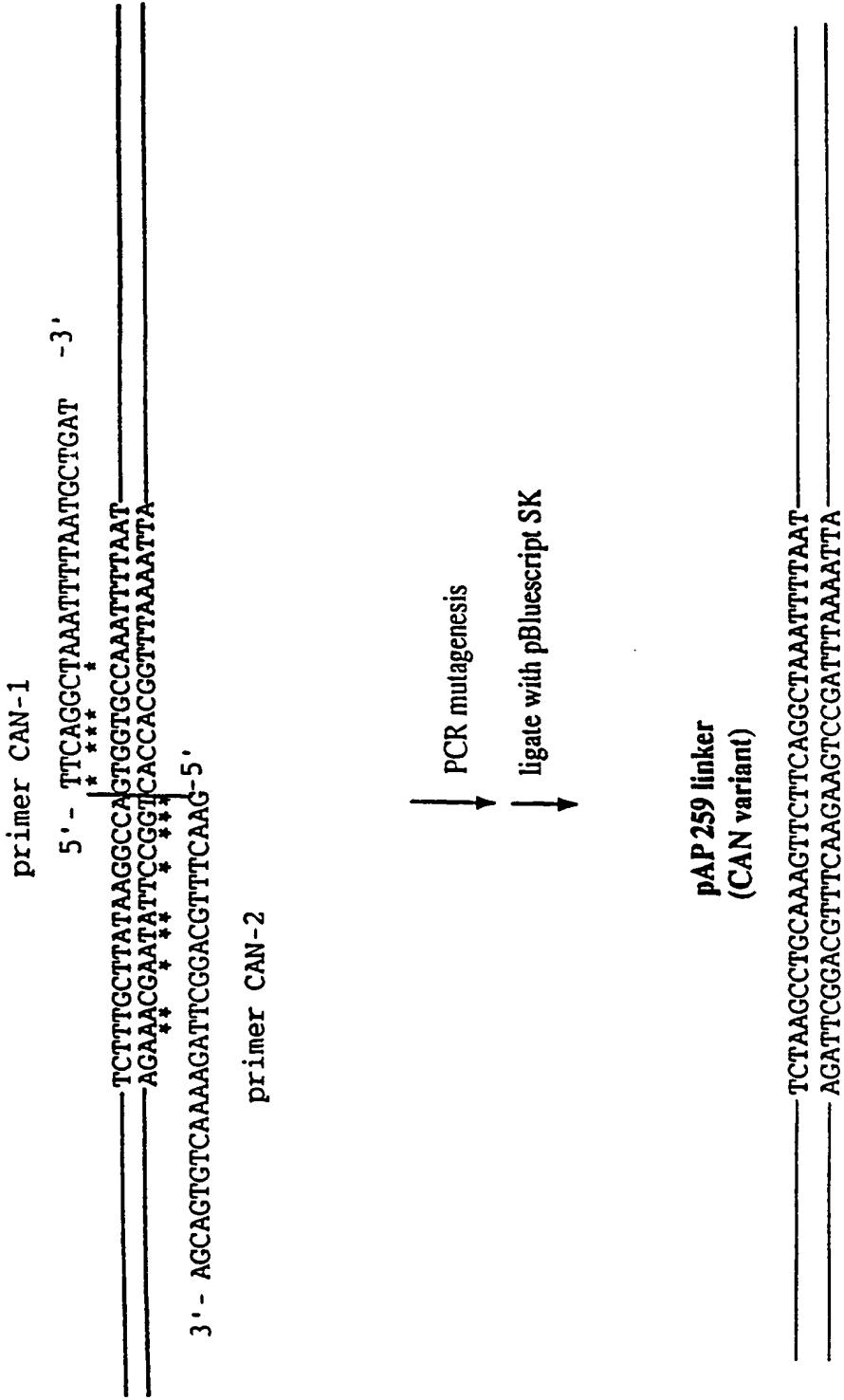
 1801 GGACATTGTAATTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAAACATTGACTTTCTGTCGTTCAATATAGCTTAAGG

 1851 TGCAG
 ACGTC

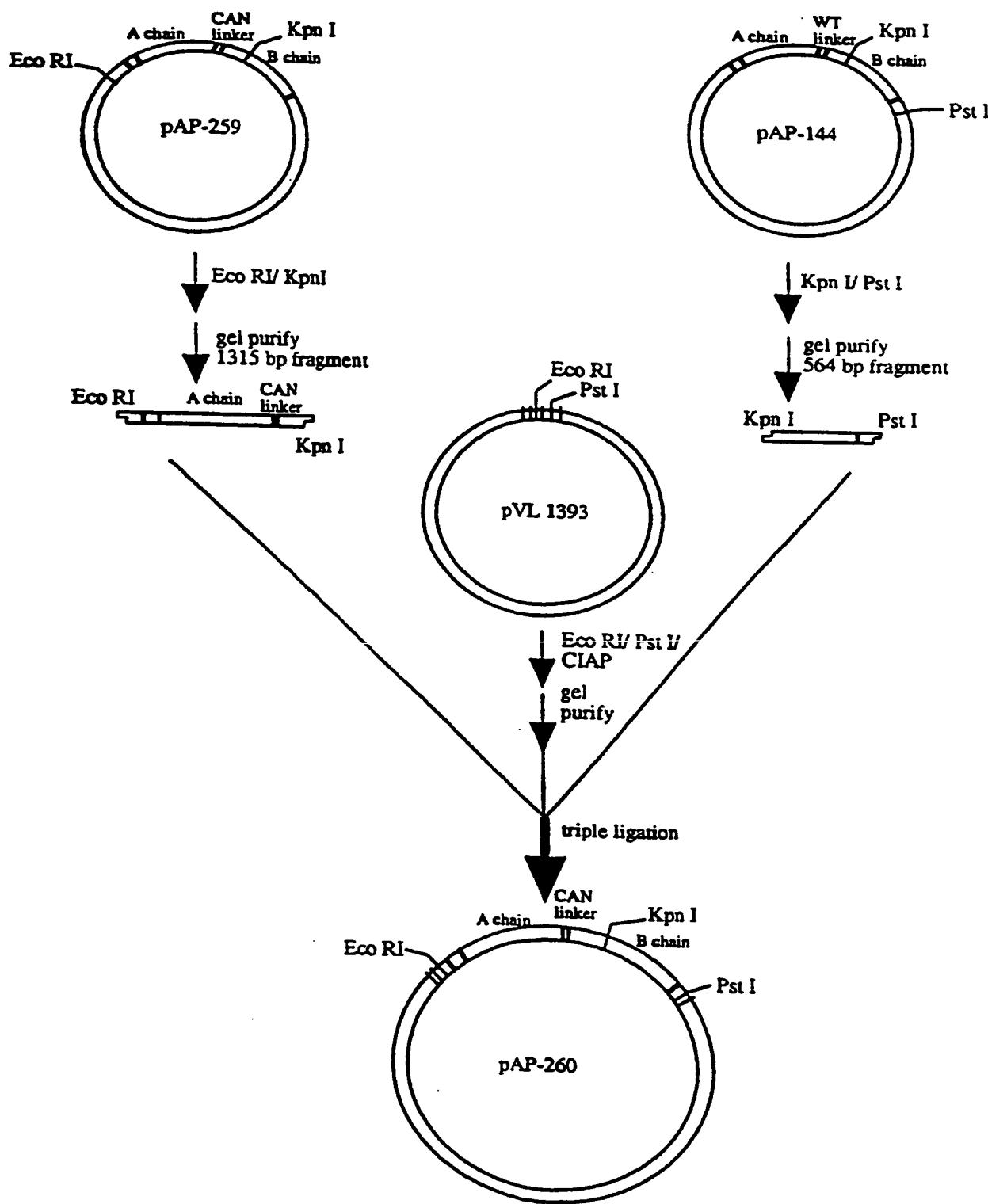
118/254

FIGURE 25A**SUBSTITUTE SHEET (RULE 26)**

119 / 254

FIGURE 25B**WT preprorin linker****SUBSTITUTE SHEET (RULE 26)**

120/254

FIGURE 25C**SUBSTITUTE SHEET (RULE 26)**

121/254

FIGURE 25D

10	20	30	40	50
1 GAATT	CTTAAGT	CTTGC	GTATG	CAGT
10 AACAC	AAGTACT	GGCCCTC	TATGATA	ACATTAC
20 ACCGGGAGG	TTGGATCC	GGGTGGT	GTACATTAG	GTACATAC
30 AAATACTATT	CACCTCAGGG	CTTTCACATTAG	GGAAAGTGTAA	CGTCA
40 TGAAT	GGGTGGGAG	GGTCTTCAC	GTGGAGTCCC	ACAGAAAGTGTAA
50 GAAACATGG	GGCTTGAT	GGTGTGGAG	GGGAGTCCC	GTAA
101 AGGATAACA	ACATATTCCCCA	AAACAATACCA	ATTATAAAC	TTTACCA
201 TCCTATTGTTG	TATAAGGGTTT	GTTATGGGTA	ATATTGAAATGGGT	GTT
151 GCGGGTGCC	ACTGTGCAAAGCT	ACACAAACTT	TATCAGAGCTG	TGCGG
251 CGCCACGGTGAC	CGTTCGATGTG	TTGAAATAGT	TCTCGACAA	AGCGCC
301 TCGTTAACAA	ACTGGAGCTGATG	TGAGACATG	ATATACCAGT	GTTGCCAA
351 AGCAAATTGTTG	ACCTCGACTAC	ACTCTGTACT	ATATGGTC	ACAAACGGTT
401 ACAGAGTTGG	TTGCCTATAAACCA	ACGGTTTATTTAG	TTGAAACTCTCA	TGTCTAACCAACGG
451 TGTCTAACCAACGG	TATTTGGGATTTG	CCAAATAAAATCA	ACTTGAGAGT	AACTTGAGAGT
501 AATCATGCAGAG	CTTCTGTTACATTAG	CGCTGGATGT	CACCAATGCATA	TTAGTACGTCTCGAAAGACAATG
551 ATCGTACCTCGG	CTACCGTGGAAATAG	CGCATATTTC	CATCCTGACA	ACACCAGCCGATGGCACG
601 351 ATCAGGAAGATG	CAGAAGCAATCA	CTCATTTCA	CTGATGTTAAAAT	TAGTCCTTCTACGTCTCGTAGT
651 401 TAGTCCTTCTACGTCTCGTAGT	AGAGACTCT	TTTATAGCT	CAACCC	TTTACCAAGTAGGACTGT
701 451 GCTATATGTAAGCGGAAACCACCA	TTAAACTATCTG	AACTATCTGAACTTGTGAA	TTGAGACT	ACG
751 501 TGGTAATCTGAGAGAAAAT	TCGAGTTGGGAAATGGT	CCACTAGAGGAGG	ACCAACTTGC	GGGAAATGGTCAACTAGAGGAGG
801 551 ACCATTAGACTCT	CTTATATTACAGT	ACTGGTGGCACTCAG	CTTCCA	CTCCTCC
851 601 GATCTGCACCA	AGATCCTAGCGTA	ATTACACTTGAGA	ATAGTTGGGGAGA	CTAGACGTGGCTAGGATCGCATT
901 651 ATTACCA	ATATTGAGGGAGAAAT	CGCACGAGAATTAGGT	ACAACCGGA	TAAAGGTTATATAACT
951 701 TAATCCCTATCATAGCT	CTCATGGGTG	TATAGATGCGC	ACCTCCACCA	TTACCGTCTCGGAAACGATCAGGTTA
1001 751 TCAACTGCAAAAGACG	TAATGGTCAAATT	CGTAATTCAAGTGT	TACGATGTGAGTA	AGTGTGACGTTCTGCAT
1051 801 AGTGTCAAAGATT	CGCTGCAAAGTT	CTTCAAGGTTAAGT	GTGACTCTTATCAACCCC	CTCT
1101 851 TATAATCCCTATCATAGCT	CTCATGGGTG	TATAGATGCGC	ACCTCCACCA	ATAATTAGGGATAGTATCGAGAGT
1151 901 TCGTCACAGTTCTAAGCCTG	CAAAGTTCTCAGG	CTAAATTGTTAAT	TTAATGCAAGAAGTCCGATT	AAATTACG

122/254

FIGURE 25D (CONT'D)

951 TGATGTTGTATGGATCCTGAGCCCATACTGCATCGTAGGTCGAATG
 ACTACAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACTACAATCCCTACCTTCTAAGGTGTTGCCCTTGCCTTAT

 1051 CAGTTGTGCCATGCAAGCTAAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTCAAGATTATGTCTACGTTAGTCGAGACCTGAAA

 1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC

 1151 GGTACAGTCGGGAGTCTATGTGATGATCTATGATTGCAAACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCACATCATAATCC
 TGACTACGGTGGCGACCGTTATACCTTATTACCTTGGTAGTATTAGG

 1251 CAGATCTAGTCTAGTTTAGCAGCGACATCAGGGAACAGTGGTACCCACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTGTCACCATGGTGTG

 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAGGTTGGCTTCTACT
 AATGTCACGTTGGTTGTAATACGGCAATCAGTTCCAACCGAAGGATGA

 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACAATGTTGTAACAACCCGATATACCAGACAC

 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGGACTGTAGCAGTGAAA
 GAACGTTCGTTATCACCTGTTACCTATCTCTGACATCGTCACTTT

 1451 AGGCTGAACAACAGTGGCTCTTATGCAAGATGGTCAATACGTCCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

 1501 CAAAACCGAGATAATTGCCCTACAAGTGATTCTAATATAACGGAAACAGT
 GTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTGTCA

 1551 TGTTAAGATCCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

 1601 TCAAGAATGATGAAACCATTAAATTGTATAGTGGATTGGTAGAT
 AGTTCTTACTACCTGGTAAATTTAAACATATCACCTAACCAATCTA

 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTACCCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGAGAGGT

 1701 TGGTGACCCAAACAAATATGGTACCAATTGGATAGACAGATTACT
 ACCACTGGGTTGGTTATACCAATGTTAATAAAACTATCTGTCTAATGA

 1751 CTCTTGCACTGTGTGTCCTGCCATGAAAATAGATGGCTAAATAAAAAA
 GAGAACGTCACACACAGGACGGTACTTTATCTACCGAATTATTTT

 1801 GGACATTGAAATTGGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTAAACATTGACTTCCCTGTCGTTCAATATAGCTTAAGG

 1851 TGCAG
 ACGTC

123/254

FIGURE 26

Ricin linker (wild type) :

A chain- S L L I R P V V P N F N -B chain

pAP-223/224 linker (MAL-A) :

A chain- Q V V Q L Q N Y D E E D -B chain

pAP-225/226 linker (MAL-B) :

A chain- L P I F G E S E D N D E -B chain

pAP-227/228 linker (MAL-C) :

A chain- Q V V T G E A I S V T M -B chain

pAP-229/230 linker (MAL-D) :

A chain- A L E R T F L S F P T N -B chain

pAP-231/pAP-232 linker (MAL-E) :

A chain- K F Q D M L N I S Q H Q -B chain

124/254

FIGURE 27

Ricin linker (wild type) :

A chain- S L L I R P V V P N F N -B chain

pAP-245/246 linker (CMV-A) :

A chain- S G V V N A S C R L A N -B chain

pAP-247/248 linker (CMV-B) :

A chain- S S Y V K A S V S P E N -B chain

pAP-233/234 linker (HERPES SIMPLEX-1 A) :

A chain- S A L V N A S S A H V N -B chain

pAP-235/236 linker (HERPES SIMPLEX-1 B) :

A chain- S T Y L Q A S E K F K N -B chain

pAP-249/250 linker (HUMAN HERPES VIRUS-6) :

A chain- S S I L N A S V P N F N -B chain

pAP-237/pAP-238 linker (VZV-A) :

A chain- S Q D V N A V E A S S N -B chain

pAP-239/pAP-240 linker (VZV-B) :

A chain- S V Y L Q A S T G Y G N -B chain

pAP-253/pAP-254 linker (ILV) :

A chain- S K Y L Q A N E V I T N -B chain

pAP-255/pAP-256 linker (HAV-A) :

A chain- S E L R T Q S F S N W N -B chain

pAP-257/pAP-258 linker (HAV-B) :

A chain- S E L W S Q G I D D D N -B chain

125/254

FIGURE 28

Ricin linker (wild type) :

A chain- S L L I R P V V P N F N -B chain

pAP-259/260 linker (CAP-A) :

A chain- S K P A K F F R L N F N -B chain

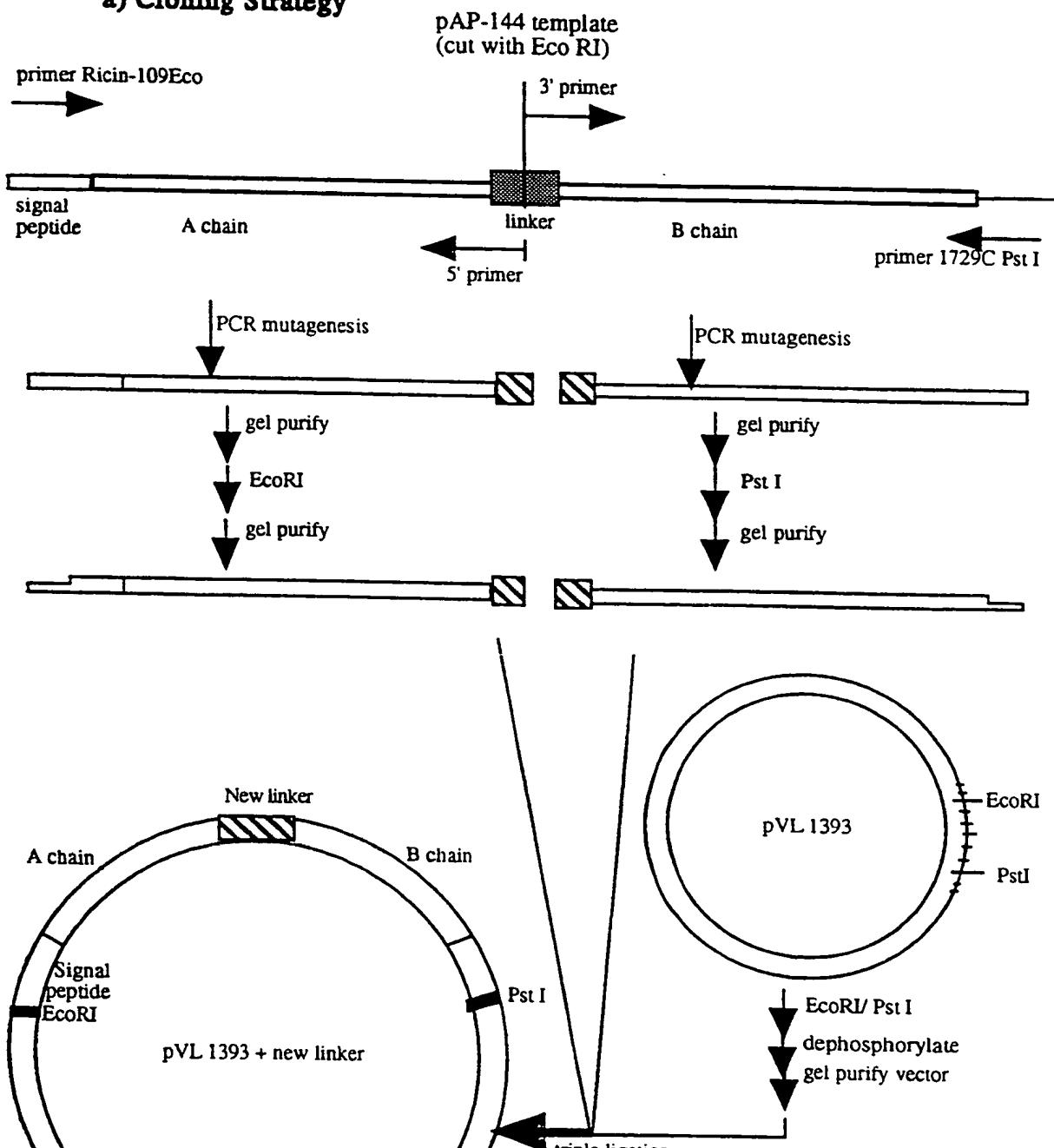
pAP-261/262 linker (CAP-B) :

A chain- S K P I E F F R L N F N -B chain

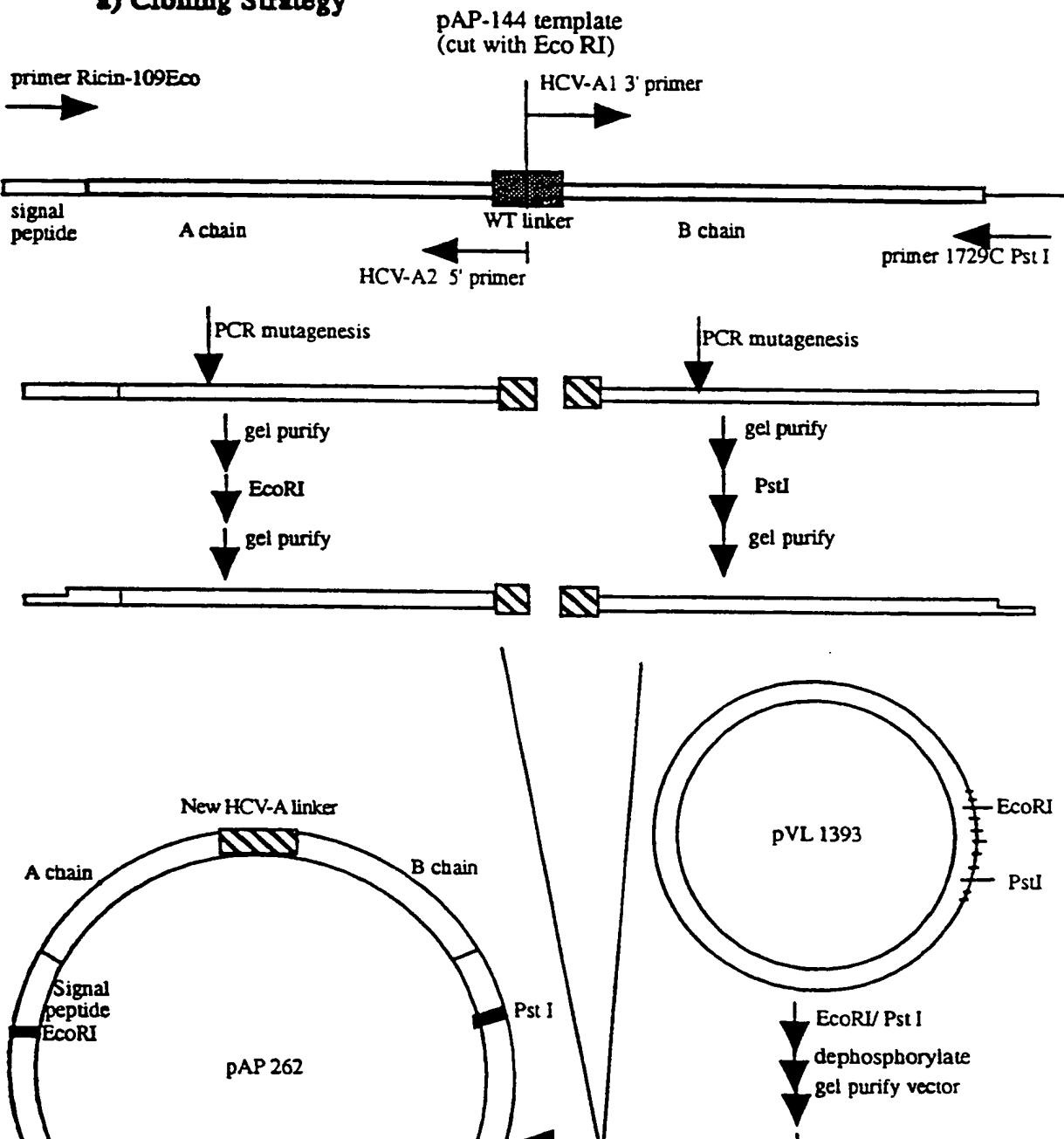
pAP-263/264 linker (CAP-C) :

A chain- S K P A E F F A L N F N -B chain

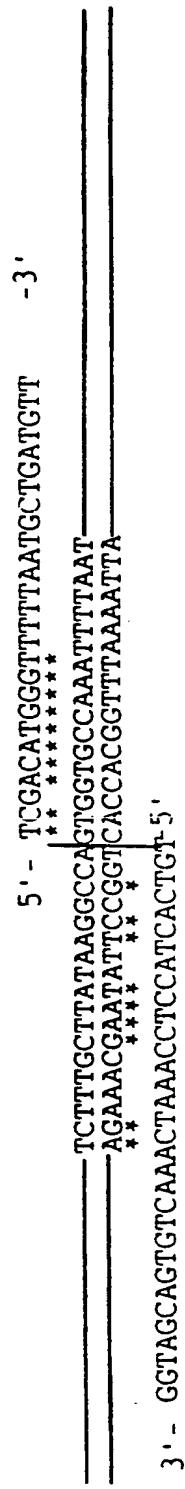
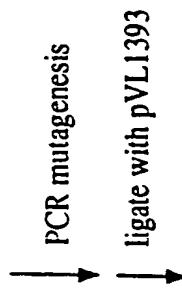
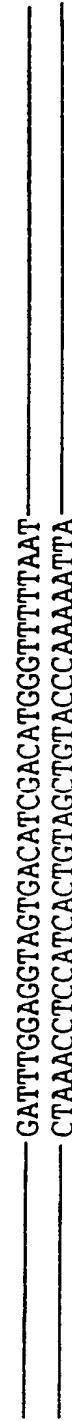
126/254

FIGURE 29**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

127/254

FIGURE 30A**PCR Mutagenesis of Preproricin Gene to Create An HCV-A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

128/254

FIGURE 30B**Sequence of HCV-A Linker Region****WT preprorin linker****primer HCV-A1****5' primer HCV-A2****pAP 262 linker
(HCV-A variant)**

129/254

FIGURE 30C (P1)

Sequence of pAP262 insert

10	20	30	40	50
1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
CTTAAGTACTTGGCCCTCCTTATGATAACATTACCTACATACGTCA				
51 GGCAACATGGCTTGTGATCCACCTCAGGGTGGCTTCACATTAG				
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGAATC				
101 AGGATAAACACATATTCCCCAAACAATACCCAAATTATAAAGTACCA				
TCCTATTGTTGTATAAGGGGTTGTTAGGTTAATATTGAAATGGTGT				
151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTGTCGCC				
CGCCCCACGGTGACACGTTGATGTGTTGAAATAGTCTGACAAGCGCC				
201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAAGTGTGCCA				
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT				
251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACCTCTCA				
TGTCTAACCAAACGGATATTGGTTGCCAAATAAAACTAACATTGAGAGT				
301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351 TGTGGTCGGCTACCGTGCTGAAATAGCGCATATTCTTCATCCTGACA				
ACACCAGCCGATGGCACGACCTTATCGCGTATAAAGAAAGTAGGACTGT				
401 ATCAGGAAGATGCAGAACACTCATCTTCACTGATGTTCAAAAT				
TAGTCCTTCTACGTCTCGTTAGTAGAAAGTAGTGAACAGTTTA				
451 CGATATACATTGGCTTGGTGTAATTATGATAGACTTGAACAACTTGC				
GCTATATGTAAGCGGAAACCACCATTAATAACTATCTGAACCTGTTGAACG				
501 TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG				
ACCATTAGACTCTCTTTATAGCTAACCTTACCAAGGTGATCTCCTCC				
551 CTATCTAGCGCTTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
GATAGAGTCGCGAAATAATAATGTATGACCGACCGTGAGTCGAAGGTTGA				
601 CTGGCTCGTTCTTATAATTGCATCCAAATGATTCAGAACAGCAAG				
GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTCGTT				
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
TAAGGTTATATAACTCCCTTTACCGTGCTTAAATCCATGTTGGCCT				
701 GATCTGCACCAAGATCCTAGCGTAATTACACTTGAGAAATAGTTGGGGAGA				
CTAGACGTGGTCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT				
751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT				
GAAAGGTGACGTAAAGTCTCAGATTGGTTCTCGGAAACGATCAGGTTA				

130/254

FIGURE 30C (P2)

801 TCAACTGCAAAGACGTAATGGTCCAAATTCACTGTGTACGATGTGAGTA
 AGTTGACGTTCTGCATTACCAAGGTTAACGACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCACATATCTACCGTGGAGGTGGT
 901 TCGTCACAGTTGATTGGAGGTAGTGACATCGACATGGGTTTTAATGC
 AGCAGTGTCAAACAAACCTCCATCACTGTAGCTGTACCCAAAAATTACG
 951 TGATGTTGTATGGATCCTGAGCCCATACTGTGCGTATCGTAGGTCGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATTCCAGCTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATAACACAACATACAATCCCTACCTTCTAAGGTGTTGCCCTTGCCTTAT
 1051 CAGTTGTCGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTCAAGATTATGTCTACGTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAATCC
 TGACTACGGTGGCGACCCTTATACCTTACCTTGGTAGTATTAGG
 1251 CAGATCTAGTCTAGTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTGTCACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTATGCCGTTAGTCAAGGTTGGCTTCCTACT
 AATGTCACGTTGGTTGTAATACGGCAATCAGTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTCGTTATCACCTGTTACACCTATCTCTGACATCGTCACTTT
 1451 AGGCTGAACAAACAGTGGCTCTTATGCAGATGGTCAATACGTCCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCCTACAAGTGTATTCTAACGAAACAGT
 GTTTGGCTCTATTACGGAATGTTCACTAAGATTATGCCCTTGTCA
 1551 TGTAAAGATCCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAAATGATGGAACCATTAAATTGTATAGTGGATTGGTAGAT
 AGTTCTTACTACCTTGGTAAAATTAAACATATCACCTAACCAATCTA

131/254

FIGURE 30C (P3)

1651 GTGAGGCATCGATCCGAGCCTAAACAAATCATTCTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTGATAGACAGATTACT
ACCACTGGGTTGGTTATACCAATGGTAATAAAACTATCTGTCTAATGA

1751 CTCTTGCACTGTGTGTGCCATGAAAATAGATGGCTTAAATAAAAAA
GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTT

1801 GGACATTGTAATTTGTAAC TGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTAAACATTGACTTCCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP262

132/254

FIGURE 30D

**-Amino Acid Sequence Comparison of Mutant
Preproricin Linker Region of HCV-A to Wild Type**

Wild type Ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-262 linker:
(HCV-A linker) A chain- D L E V V T S T W V F N -B chain

133/254

FIGURE 31A

PCR Mutagenesis of Preproricin Gene to Create An HCV-B Variant Gene in Baculovirus Transfer Vector, pVL 1393

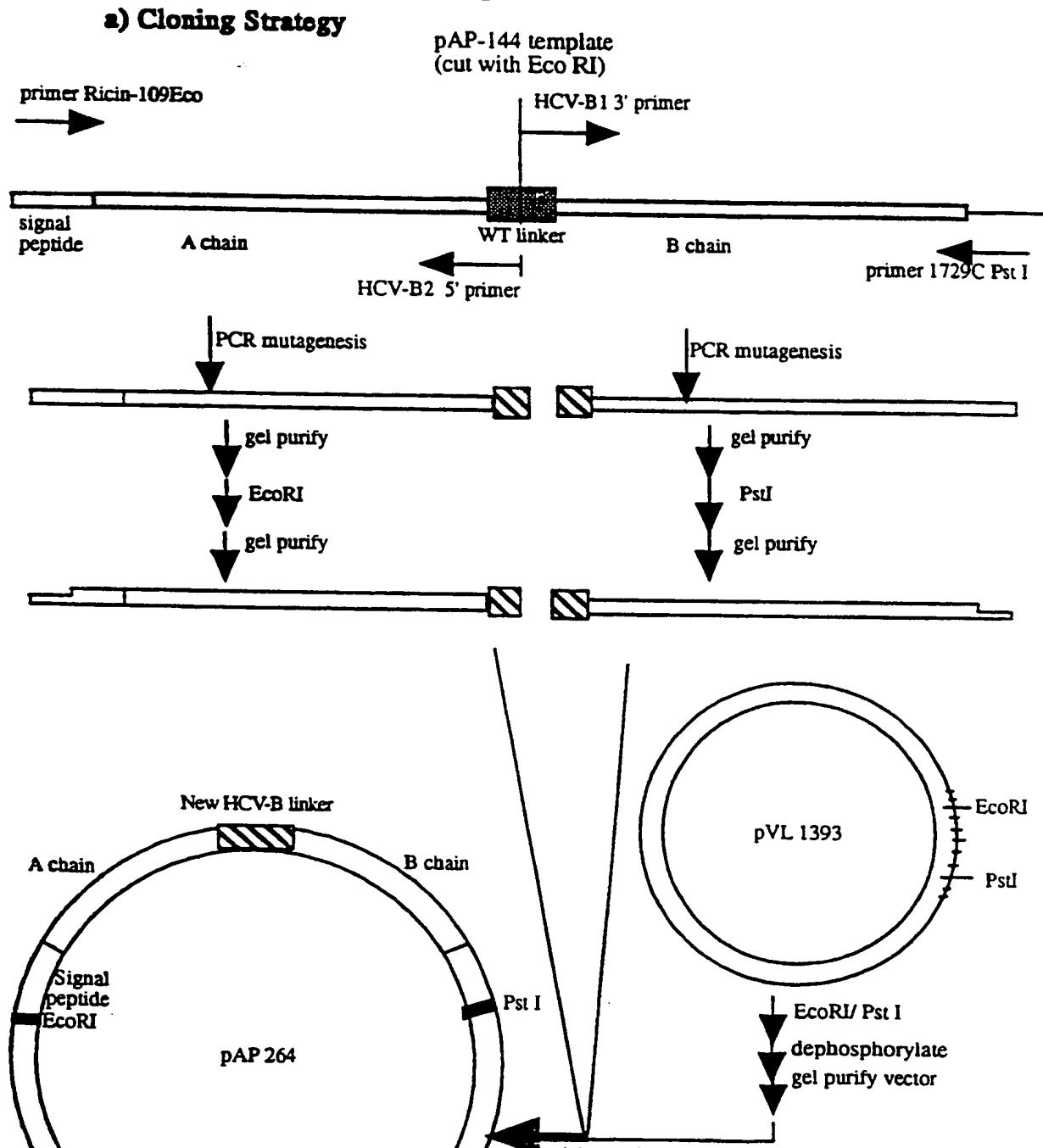
**SUBSTITUTE SHEET (RULE 26)**

FIGURE 31B

Sequence of HCV-B Linker Region

wT preprorocin linker

primer HCV-B1

5' - CGCTCACACCTTTAATGCTGATCTT
 * * * * * * * *
 TCTTGCTTAAAGGCCAGTGGTGC~~CA~~AATTAAAT
 AGAAACGAATATTCCGG~~T~~ACACCGGTTAAATTA
 * * * * * * * * * *
 3' - GGTAGCAGTGTCAA~~ACT~~TACCTTCACA-5'
 -3'

5' primer HCV-B2

```

graph TD
    A[pVLL139] --> B[PCR mutagenesis]
    B --> C["ligate with pVLL139"]

```

pAP264 linker
(ICV-B) variant

- GATGAGATGGAAAGAGTGTGGCTCACACCTTTAAT-
- CTACTCTACCTTCTACACGGCAGTGTGAAAAAATTA-

135/254

FIGURE 31C (P1)

Sequence of pAP264 insert

10	20	30	40	50
1 GAATTCA	TTGAAACCGGGAGGA	ATACTATTGT	AATATGGATGT	TATGCAGT
CTTAAGTACT	TTGGCCCTC	TTATGATA	ACATTAC	CTACATACGTCA
51 GGCAACATGG	CTTGTTGGATCC	CACCTCAGGGTGG	TTCACATTAG	
CCGTTGTACCGAA	ACAAAACCTAGGTGG	AGTCCCACCAGAA	AGTGTAA	TC
101 AGGATAACA	ACATATTCCCCAA	ACAATACCCAATT	TAAAC	TTACCA
TCCTATTGTTG	TATAAGGGTTG	TATGGGTTA	ATATTG	AAATGGGT
151 GCGGGTGCC	ACTGTGCAAAG	CTACACAAACTT	TACAGAGCTG	TTCGCCG
CGCCCACGGT	GACACGTTG	ATGTGTTG	AAATAGT	CTCGACAAGCG
201 TCGTTAACAA	ACTGGAGCTG	ATGTGAGACATG	ATACCA	AGTGTGCCAA
AGCAAATTGTTG	ACCTCGACTAC	ACTCTG	ACTATATGGT	CACAACGGTT
251 ACAGAGTTGG	TTGCCTATAAACCA	ACGGTTATTG	TTAGTTG	AACTCTCA
TGTCTAACCA	ACGGATATTG	GGTTGCCAA	AAATAA	ACTCAACTGAGAGT
301 AATCATGCAGAG	CTTCTGTTACATTAG	GCGCTGGATGT	CACCAATGCATA	
TTAGTACGTCTC	GAAAGACAATG	TAAATCGCGAC	CTACAGTGGT	TACGTAT
351 TGTGGTCGG	TACCGTGTGG	AAATAGCGC	ATATTCTTC	CATCCTGACA
ACACCAGCCG	ATGGCACG	ACCTTATCGCG	TATAAAGAA	AGTAGGACTGT
401 ATCAGGAAGAT	GCAGAACGCAAT	CACTCATCTTCA	CTGATGTT	AAAAT
TAGTCCTTCTAC	GTCTTC	GTGAGTAGA	AAAAGT	GACTACAAGTTA
451 CGATATA	CATTGCCTTGG	TTGTAATTATG	ATAGACTTG	AAACAAC
GCTATATG	TAAGCGGAA	ACCACCA	TAAACTATCTG	AACTGTTGAA
501 TGGTAATCTG	AGAGAAAAAT	ATCGAGTTGG	AAATGGTCC	ACTAGAGGAGG
ACCATTAGACT	CTCTTT	TATAGCTCA	ACCCTTAC	CAAGGTGATCTCCTCC
551 CTATCTCAGCG	CTTTATTATTAC	AGTACTGGTGG	CACTCAGCTT	CCAACT
GATAGAGTC	CGCAAATAA	ATGTCA	GTGACCACCG	TGAGTCGAAGGTTGA
601 CTGGCTCG	TCTTATAAATTG	CATCCAAATG	ATTTCAGAAG	CAGCAAG
GACCGAGCA	AGGAAATTAA	ACGTAGGTT	ACTAAAGT	CTCGTCGTT
651 ATTCCAAT	TATTGAGGGAGA	AAATGCGC	ACGAGAAATTAGGT	ACAACCGGA
TAAGGTT	AAATTA	ACTCC	CTTAC	CGTGTCTTA
701 GATCTGCACC	AGATCCTAG	CGTAATTAC	ACTTGAGA	AAATAGTTGGGGAGA
CTAGACGTGG	TCTAGGATCG	CATTAATGT	GAAC	TCTTATCAACCCCTCT
751 CTTTCCACT	GCATTCAAGAG	TCTAACCA	AGGAGC	TTGCTAGTCCAAT
GAAAGGTGAC	GTAAAGTTCTC	AGATTGGT	CCCTCG	AAACGATCAGGTTA

136/254

FIGURE 31C (P2)

801 TCAACTGCAAAGACGTAATGGTCAAATTCAAGTGTGTACGATGTGAGTA
 AGTTGACGTTCTGCATTACCAAGGTTAACATCACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCTCATGGTGTAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACGCGTGGAGGTGGT
 901 TCGTCACAGTTGATGAGATGGAAGAGTGTGCGTCACACCTTTAATGC
 AGCAGTGTCAAACACTACTCTACCTTCAACACGCAAGTGTGGAAAAATTACG
 951 TGATTTGTATGGATCCTGAGCCCATAAGTGCATCGTAGGTCGAAATG
 ACTACAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACTACCCATACCTTAAGGTGTTGCCTTGCCTTAC
 1051 CAGTTGTGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTCAAGATTGTCTACGTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
 CTTTCTCTGTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCGCTGGCAAATATGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCTTACCTTGGTAGTATTAGG
 1251 CAGATCTAGCTAGTTAGCAGCGACATCAGGAAACAGTGGTACCAACAC
 GTCTAGATCAGATAACGTCGCTGTAGTCCCTGTCACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTATGCCGTTAGTCAGGTTGGCTTCACT
 AATGTCACGTTGGTGTAAATACGGCAATCAGTTCAACCGAAGGATGA
 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAAACACCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGGACTGTAGCAGTGA
 AACGTTCGTTATCACCTGTTACACCTATCTCCTGACATCGTCACTT
 1451 AGGCTGAACAAACAGTGGCTCTTATGCAGATGGTCAATACGTCCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCCTACAAGTGATTCTAATATACGGAAACAGT
 GTTTGGCTCTATTACGGAATGTTCACTAAGATTATATGCCCTTGTCA
 1551 TGTAAAGATCCTCTTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTAAATTGTATAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTTGGTAAAATTAAACATATCACCTAACCAACATCTA

137/254

FIGURE 31C (P3)

1651 GTGAGGCAGTCGGATCCGAGCCTAAACAAATCATCTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTGATAGACAGATTACT
ACCACTGGGTTGGTTATACCAATGGTAATAAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTCTGCCATGAAAATAGATGGCTAAATAAAAAA
GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATT

1801 GGACATTGTAATTTGTAAGTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTAAAACATTGACTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP264

138/254

FIGURE 31D

**-Amino Acid Sequence Comparison of Mutant
Preproricin Linker Region of HCV-B to Wild Type**

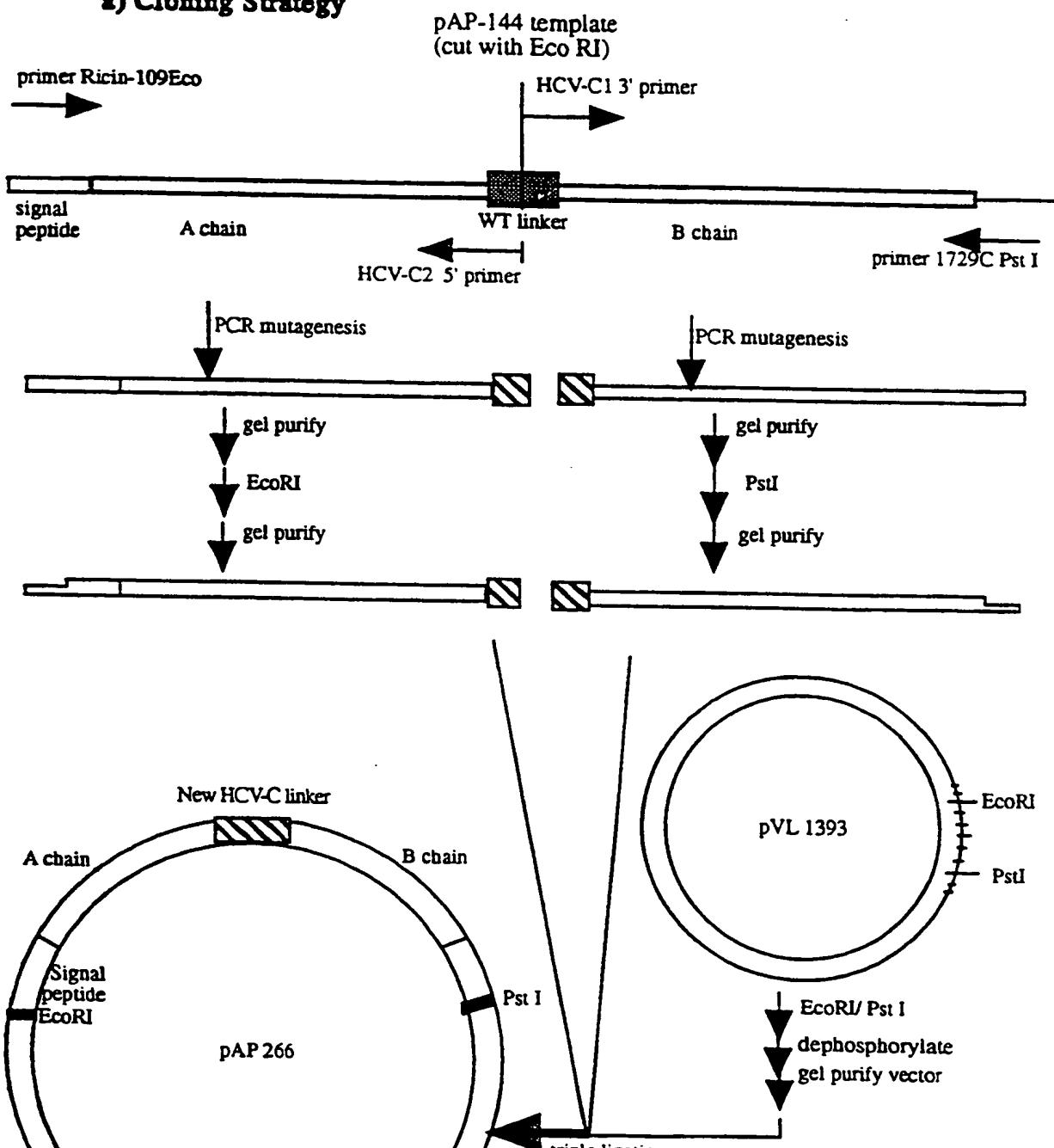
Wild type Ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-264 linker:
(HCV-B linker) A chain- D E M E E C A S H L F N -B chain

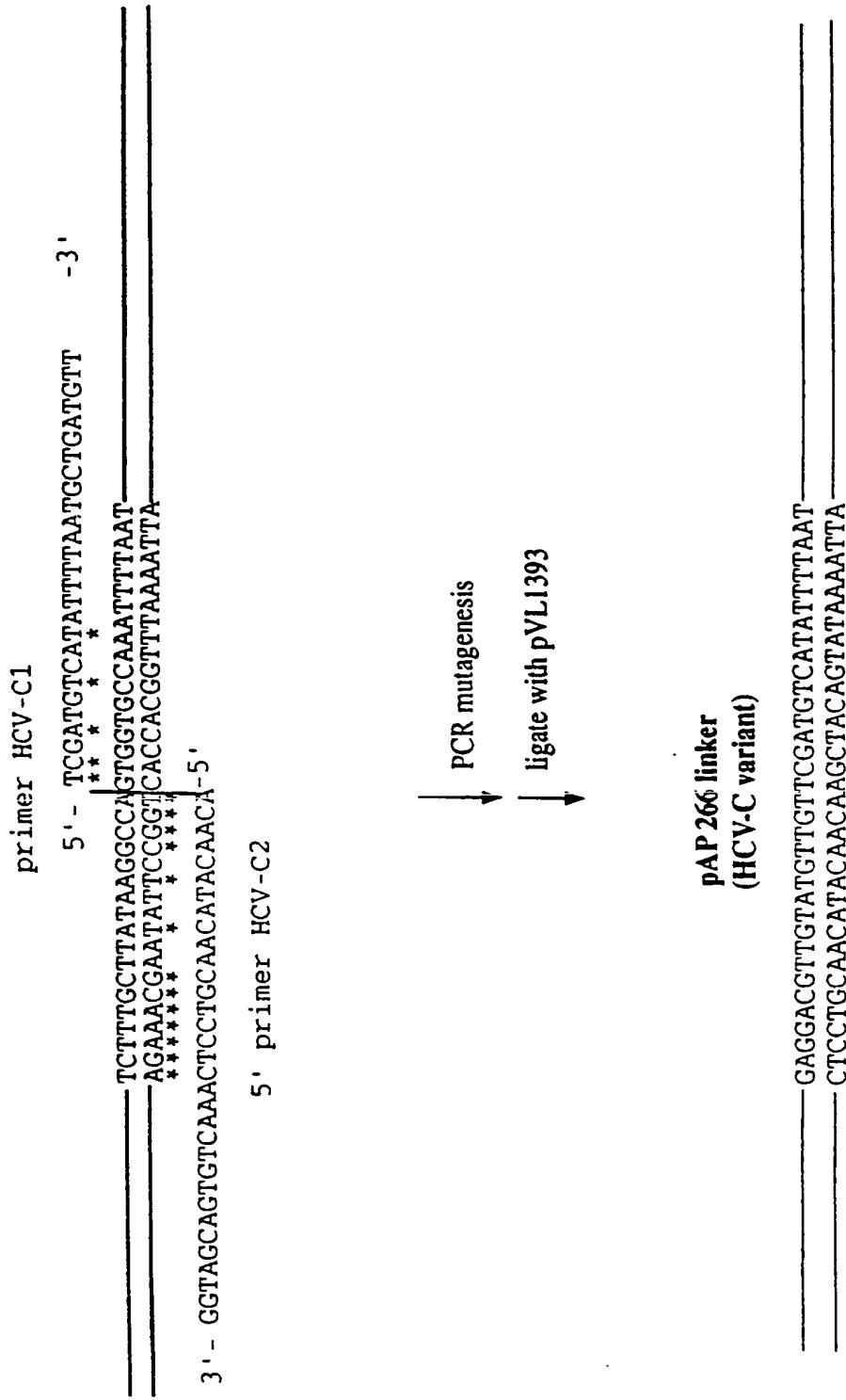
139/254

FIGURE 32A

- PCR Mutagenesis of Preproricin Gene to Create An HCV-C Variant Gene in Baculovirus Transfer Vector, pVL 1393

a) Cloning Strategy**SUBSTITUTE SHEET (RULE 26)**

140/254

FIGURE 32B**Sequence of HCV-C Linker Region****WT preproycin linker**

141/254

FIGURE 32C (P1)

Sequence of PAP266 insert

10	20	30	40	50
1 GAATTCAATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT CTTAAGTACTTGCCCTCCTTATGATAACATTACACATACAGTCA				
51 GGCAACATGGCTTGTGATCCACCTCAGGGTGGCTTTCACATTAG CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC				
101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTACCA TCCTATTGTTGATAAGGGTTGTTATGGGTAATATTTGAAATGGTGT				
151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTGTTCGCG CGCCCACGGTGACACGTTGATGTGTTGAAATAGTCGACAAAGCGCC				
201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATAACCAGTGGTGC AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT				
251 ACAGAGTTGGTTGCCTATAAAACCAACGGTTATTAGTTGA TGTCTCAACCAACGGATATTGGTGCAAATAAAACTGAGAGT				
301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGC TAGTACGTCTCGAAAGACAATGTAATCGCACCTACAGTGGTACGTAT				
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTCTTCATCCTGACA ACACCAAGCCGATGGCACGACCTTATCGCGTATAAAGAAAGTAGGACTGT				
401 ATCAGGAAGATGCAGAACGAAATCACTCATCTTCACTGATGTC TAGTCCTCTACGTCTCGTTAGTAGAAAGTGA CTACAAGTTTA				
451 CGATATACATTGCCTTGGTGGTAATTATGATAGACTTG GCTATATGTAAGCGGAAACCACCATTAATAACTATCTGA ACTTGGTGAACCG				
501 TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCC ACCATTAGACTCTTTATAGCTCAACCCTTACCA GGTGATCTCCTCC				
551 CTATCTAGCGCTTATTACAGTACTGGTGGCACTCAGCT GATAGAGTCGCGAAATAATAATGTC ATGACCACCGTGAGTCGAAGGTTGA				
601 CTGGCTCGTTCTTATAATTG GCATCCAAATGATTCAGAACAGCAAG GACCGAGCAAGGAAATATTAACG TAGGTTACTAAAGTCTCGTCTTC				
651 ATTCCAATATATTGAGGGAGAAATGCG CACGAGAATTAGGTACAACCGGA TAAGGTTATATAACTCCCT CTTACCGTGCTCTTAATCC ATGTTGGCCT				
701 GATCTGCACCA GAGATCCTAGCGTAATTACACT TGAGAATAGTTGGGGAGA CTAGACGTGGTCTAGGATCG CATTAATGTA ACTCTTATCA ACCCCTCT				
751 CTTCCACTGCAATTCAAGAGTCTAACCA AGGAGCCTTGCTAGTCCA ATGAAAGGTGACGT TAAGTTCTCAGATTGG TCCCTCGGAAACG ATCAGGTTA				

142/254

FIGURE 32C (P2)

801 TCAACTGCAAAGACGTAATGGTCCAAATTCACTGTGTACGATGTGAGTA
 AGTTGACGTTCTGCATTACCAAGGTTAACGTCACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCTCATGGGTATAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACCGGTGGAGGTGGT
 901 TCGTCACAGTTGAGGACGTTGTATGTTGTCATGTCATATTTAATGC
 AGCAGTGTCAAACCTCTGCAACATACAACAAGCTACAGTATAAAAATTACG
 951 TGATGTTGTATGGATCCTGAGCCCAGTGCCTAGTAGGTCGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACAATCCCTACCTTCTAAAGGTGTTGCCTTGCCTTAT
 1051 CAGTTGTGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTAGATTATGTCTACGTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCGCTGGCAAATATGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCTTACCTGGTAGTATTAGG
 1251 CAGATCTAGTCTAGTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTGTCACCATGGTGTG
 1301 TTACAGTGCACCAACATTATGCCGTTAGTCAGGTTGGCTCCTACT
 AATGTCACGTTGGTTGTAACGGCAATCAGTCCAAACGAAGGATGA
 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTCGTTATCACCTGTTACACCTATCTCCTGACATCGTCACTT
 1451 AGGCTGAACAAACAGTGGCTTTATGCAGATGGTCAATACTGTCCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCCTACAAGTGATTCTAATATACGGGAAACAGT
 GTTTGGCTCTATTAACGGAATGTTCACTAAGATTATGCCCTTGTCA
 1551 TGTTAAGATCCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTAAATTGTATAGTGGATTGGTGTAGAT
 AGTTCTACTACCTGGTAAATTAACATATCACCTAACCAACAACTA

143/254

FIGURE 32C (P3)

1651 GTGAGGCATCGGATCCGAGCCTTAAACAAATCATTCTTACCCCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTGATAGACAGATTACT
ACCACTGGGTTGGTTATACCAATGGTAATAAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGCCTGCCATGAAAATAGATGGCTAAATAAAAAA
GAGAACGTCACACACAGGACGGTACTTTATCTACCGAATTATTTT

1801 GGACATTGTAATTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTAAACATTGACTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP266

144/254

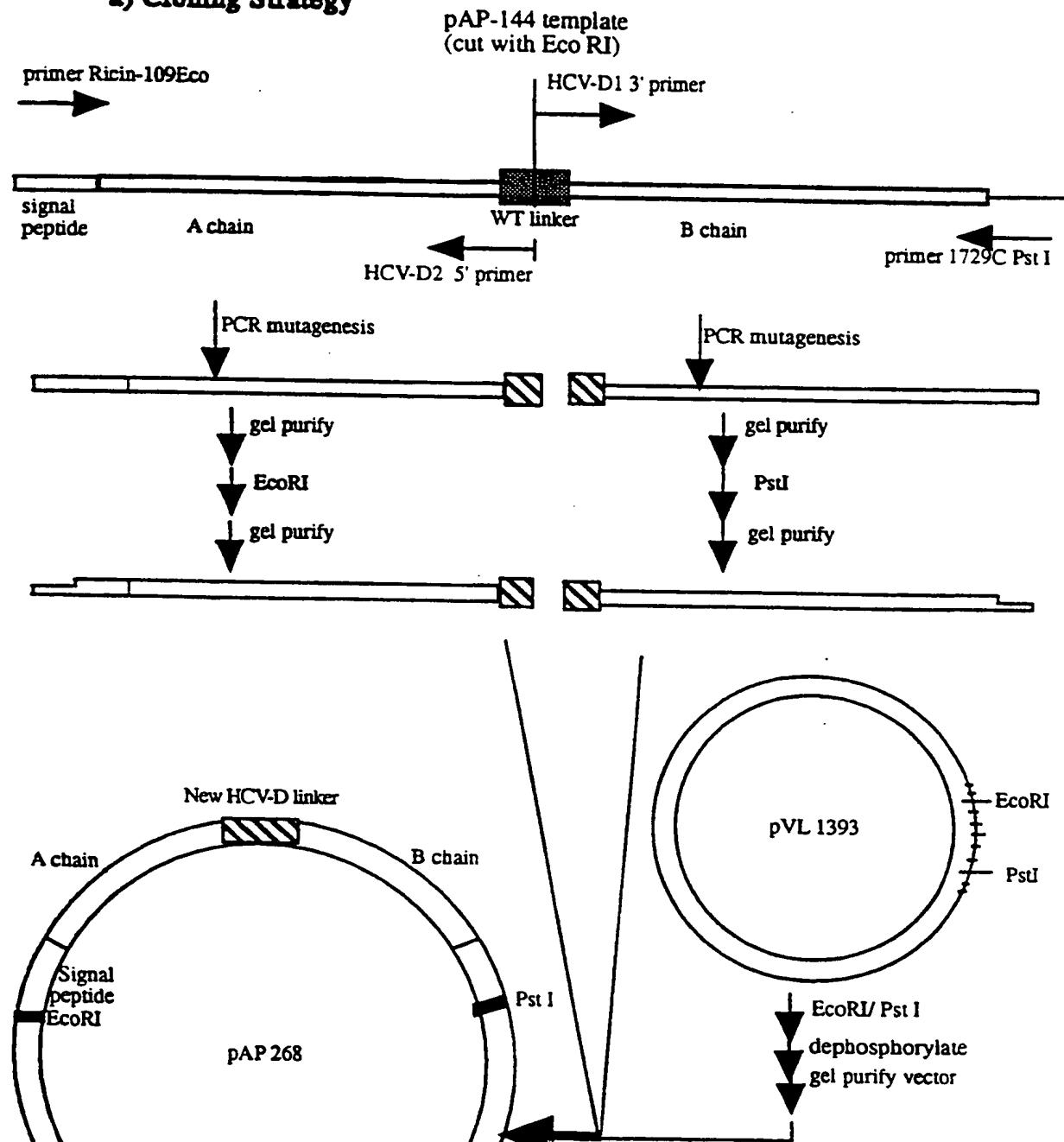
FIGURE 32D

**-Amino Acid Sequence Comparison of Mutant
Preproricin Linker Region of HCV-C to Wild Type**

Wild type Ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-266 linker:
(HCV-C linker) A chain- E D V V C C S M S Y F N -B chain

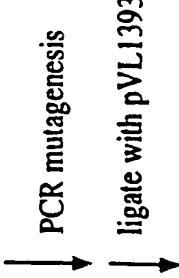
145/254

FIGURE 33A**PCR Mutagenesis of Preproricin Gene to Create An HCV-D Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

146/254

FIGURE 33B**Sequence of HCV-D Linker Region****WT prprotein linker****primer HCV-D1**

5' - GCGCCAATAACTGCTTATGCTGATGTTGGTATG - 3'
 * * * * *
 |
 TCTTGCTTATAAGGCCAAGTGGTGCCAAATTAAAT
 |
 AGAACGAATATTCCGGTACCCACGGTTAAATA
 * * * * * * * *
 3' - GGTAGCACTGTCAAATTCCCCACTCTAACGAT-5'

5' primer HCV-D2**pAP 268 linker
(HCV-D variant)**

AAGGGTGGAGATTGCTAGGGCCAATAACTGCTTAT
 TTCCCCACCTCTAACGATCGGGTATGACGAATA

147/254

FIGURE 33C (P1)

Sequence of pAP268 insert

10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAATACTATTGTAATATGGATGTATGCAGT			
	CTTAAGTACTTTGCCCTCCTTATGATAACATTACCTACATACGTCA			
51	GGCAACATGGCTTGTGATCCACCTCAGGGTGGTCTTCACATTAG			
	CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGAATC			
101	AGGATAACAACATATTCCCCAAACAATACCAATTATAAACTTTACCA			
	TCCTATTGTTGATAAGGGTTGTTATGGGTTAATATTGAAATGGTGT			
151	GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTGTTCGCGG			
	CGCCCACGGTGACACGTTCGATGTGTTGAAATAGTCTCGACAAGCGCC			
201	TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAAGTGTGCCAA			
	AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT			
251	ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACCTCA			
	TGTCTCAACCAACGGATATTGGTTGCCAATAAAATCAACTTGAGAGT			
301	AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA			
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT			
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTCTTCATCCTGACA			
	ACACCAGCCGATGGCACGACCTTATCGGTATAAAGAAAGTAGGACTGT			
401	ATCAGGAAGATGCAAGCAATCACTCATTTCACTGATGTTCAAAAT			
	TAGTCCTTCTACGTCTCGTTAGTAGAAAGTAGTGAACACTACAAGTTTA			
451	CGATATACTCGCTTGGTGGTAATTATGATAGACTTGAACAACTTGC			
	GCTATATGTAAGCGGAAACCACCATTAATAACTATCTGAACCTGTTGAACG			
501	TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG			
	ACCATTAGACTCTCTTATAGCTAACCTTACCAAGGTGATCTCCTCC			
551	CTATCTCAGCGCTTATTACAGTACTGGTGGCACTCAGCTTCCA			
	GATAGAGTCGCGAAATAATAATGTATGACCAACCGTGAGTCGAAGGTTGA			
601	CTGGCTCGTCTTATAATTGATCCAAATGATTCAGAAGCAGCAAG			
	GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTGTTC			
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA			
	TAAGGTTATATAACTCCCTCTTACCGTGTCTTAATCCATGTTGGCCT			
701	GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA			
	CTAGACGTGGTCTAGGATCGCATTATGTGAACCTTATCAACCCCCCTCT			
751	CTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCAAT			
	GAAAGGTGACGTTAAGTCTCAGATTGGTCTCGGAAACGATCAGGTTA			

148/254

FIGURE 33C (P2)

801 TCAACTGCAAAGACGTAATGGTCCAAATTCAAGTGTACGATGTGAGTA
 AGTTGACGTTCTGCATTACCAAGGTTAACATCACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACGCGTGGAGGTGGT
 901 TCGTCACAGTTAAGGGTGGAGATTGCTAGGCCAATAACTGCTTATGC
 AGCAGTGTCAAATTCCCCACCTCTAACGATCGCGTTATTGACGAATACG
 951 TGATGTTGTATGGATCCTGAGCCCAGTGCGTATCGTAGGTCGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC
 1001 GTCTATGTGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATAACACAACATACAATCCCTACCTTCTAACGGTGTGCCTTGCCTTAT
 1051 CAGTTGTGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTCAAGATTGTCTACGTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAAATGGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATAACTACTAGATACTAACGTATGACGACGT
 1201 ACTGATGCCACCGCTGGCAAATATGGATAATGGAACCACATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCTTACCTGGTAGTATTAGG
 1251 CAGATCTAGTCTAGTTAGCAGCGACATCAGGGAACAGTGGTACACAC
 GTCTAGATCAGATAACCGTCTGTAGTCCCTGTACCATGGTGT
 1301 TTACAGTGCAAACCAACATTATGCCGTTAGTCAGGTTGGCTTCACT
 AATGTCACGTTGGTTGTAACACGGCAATCAGTCCAAACGAAGGATGA
 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGCTGTG
 TTATTATGTGTTGGAAAACATGTTGGTAACAACCCGATATACCAAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTCGTTATCACCTGTCATACCTATCTCCTGACATCGTCACTTT
 1451 AGGCTGAACAACAGTGGCTCTTATGCAGATGGTCAATACGTCTCAG
 TCCGACTTGTGTCACCCGAGAAACACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCCTACAAGTGTGATTCTAACGGAAACAGT
 GTTTGCGCTCTATTACGGAATGTTCAACTAAGATTATATGCCCTTGTCA
 1551 TGTTAAGATCCTCTTGTGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCCGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTAAATTGTATAGTGGATTGGTAGAT
 AGTTCTTACTACCTGGTAAACATATCACCTAACCAACATCTA

149/254

FIGURE 33C (P3)

1651 GTGAGGCATCGGATCCGAGCCTAAACAAATCATCTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT
1701 TGGTACCCAAACCAAATATGGTTACCATATTGATAGACAGATTACT
ACCACTGGGTTGGTTATACCAATGGTAATAAAACTATCTGTCTAATGA
1751 CTCTTCAGTGTGTGTCTGCCATGAAAATAGATGGCTAAATAAAAAA
GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATT
1801 GGACATTGTAATTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTAAAACATTGACTTCCTGTCGTTCAATATAGCTTAAGG
1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP268

150/254

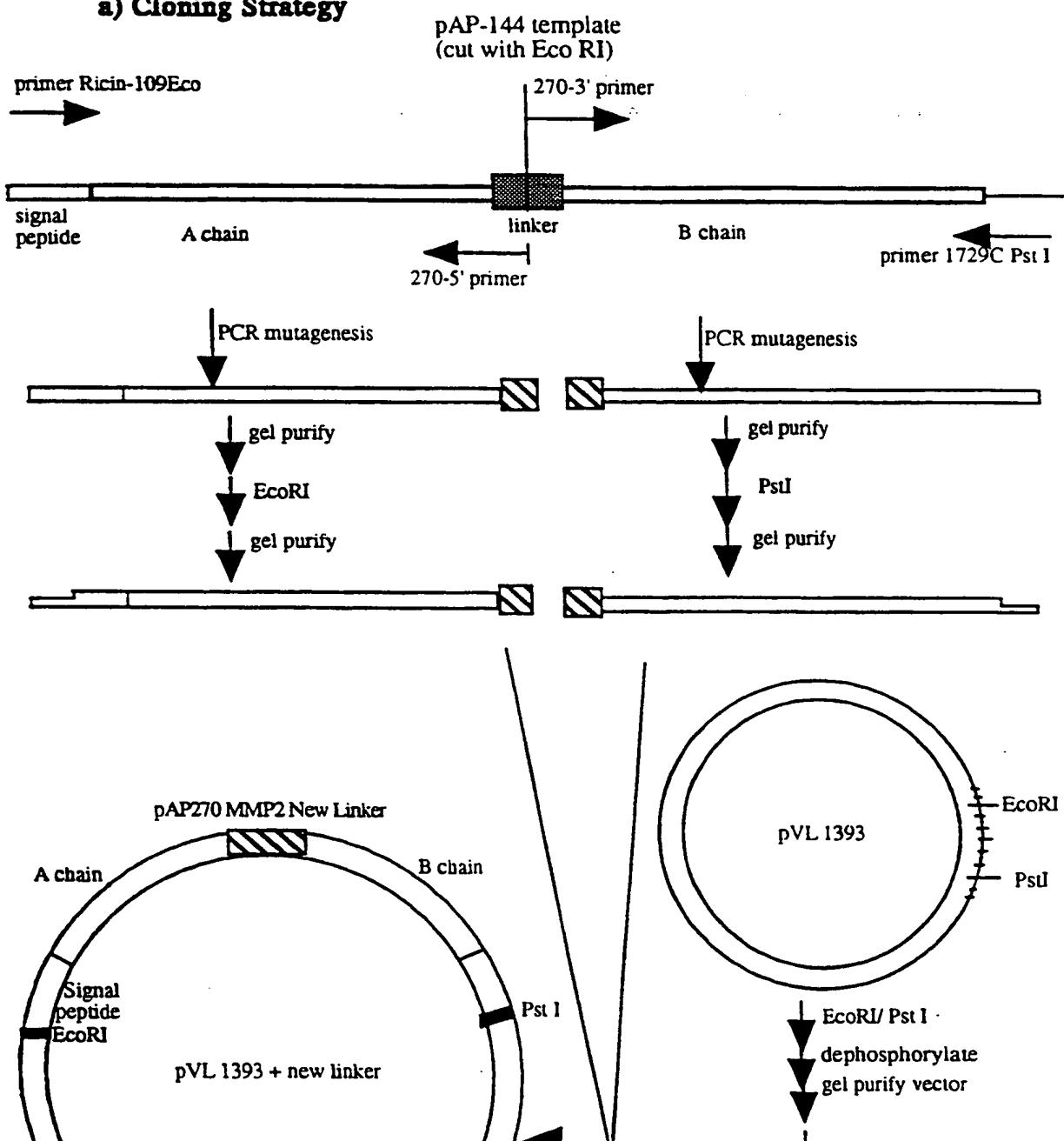
FIGURE 33D

**-Amino Acid Sequence Comparison of Mutant
Preproricin Linker Region of HCV-D to Wild Type**

Wild type Ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-268 linker:
(HCV-D linker) A chain- K G W R L L A P I T A Y -B chain

151/254

FIGURE 34A**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy****SUBSTITUTE SHEET (RULE 26)**

152/254

FIGURE 34B**Sequence of MMP-2 Linker Region****WT preprocin linker**

primer 270-3'
5' - TGGGCTCCTAATTTAATGCTGATGTTGT - 3'
| * * * *
----- TCTTGCTTATAAGGCCA | GTGGTACCAAATTTAAT -----
----- AGAACGAATATTCCGGT | CACCATGGTTAAAATTA -----
* * * * *
3' - AGCAGTGTCAAAAGAAAACGGGGACCCAAAT - 5'
primer 270-5'

1) PCR mutagenesis

2) Ligate with pVL1393

**pAP 270 linker
(MMP-2 variant)**

----- TCTTGCCCCCTGGGTTA | TGGGCTCCTAATTTAAT -----
----- AGAACGGGGACCCAAAT | ACCCGAGGATTAAAATTA -----

153/254

FIGURE 34C (P1)

Sequence of pAP270 insert

10	20	30	40	50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
 CTTAAGTACTTTGCCCTCCTTATGATAACATTATACCTACATACGTCA
 51 GGCAACATGGCTTGTTGGATCCACCTCAGGGTGGCTTACATAG
 CGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTATC
 101 AGGATAAACACATATTCCCCAAACAATACCCAATTATAAACTTACCA
 TCCTATTGTTGTATAAGGGTTGTTATGGGTTAATATTTGAAATGGTGT
 151 GCGGGTGCCACTGTGCAAAGCTACACAAAACCTTATCAGAGCTGTCGCC
 CGCCCACGGTGACACGTTGATGTGTTGAAATAGTCTGACAAGCGCC
 201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT
 251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACCTCTCA
 TGTCTCAACCAACGGATATTGGTTGCCAAATAACTCAACTTGAGAGT
 301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
 351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTCTTACCTGACA
 ACACCAGCCGATGGCACGACCTTATCGGTATAAGAAAGTAGGACTGT
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTCACTGATGTTCAAAAT
 TAGTCCTCTACGTCTCGTTAGTGAGTAGAAAGTGAACACTAAGTTTA
 451 CGATATACATTGCCTTGGTGGTAATTATGATAGACTTGAACAACTTGC
 GCTATATGTAAGCGAAACCACCATTAATACTATCTGAACCTGTTGAACG
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG
 ACCATTAGACTCTCTTATAGCTCAACCCCTTACCAAGGTGATCTCCTCC
 551 CTATCTAGCGCTTTATTACAGTACTGGTGGCACTCAGCTTCAACT
 GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
 601 CTGGCTCGTCTTATAATTGCATCCAATGATTGAGAAGCAGCAAG
 GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTCGTT
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
 TAAGGTTATATAACTCCCTTTACGGCGTCTTAACTGATGTTGGCCT

154/254

FIGURE 34C (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA
CTAGACGTGGTAGGATCGCATTAAATGTGAACCTTATCAACCCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTCCAAATTCACTGTGTACGATGTGAGTA
AGTTGACGTTCTGCATTACCAAGGTTAACGACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTCTTGCCCCCTGGGTTATGGGCTCCTAATTAAATGC
AGCAGTGTCAAAAGAACGGGACCCAAATACCCGAGGATTAAAATTACG

951 TGATGTTGTATGGATCCTGAGCCCAGTGCCTAGTGTAGGTCGAAATG
ACTACAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATAACACAACATACTACAAATCCCTACCTTCTAAGGTGTTGCCTTGCCTTAT

1051 CAGTTGTGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTAGATTATGTCTACGTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATAACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGCGACCCTTATACCTTACCTGGTAGTATTAGG

1251 CAGATCTAGTCTAGTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTGTCACCATGGTGTG

1301 TTACAGTCAAACCAACATTATGCCGTTAGTCAAGGTTGGCTTCTACT
AATGTCACGTTGGTTGTAACACGGCAATCAGTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTGTTACAACCATGTTGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATAACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTAAA
GAACGTTGTTATCACCTGTTACCTATCTCCTGACATCGTCACCTT

155/254

FIGURE 34C (P3)

1451 AGGCTGAACAAACAGTGGCTCTTATGCAGATGGTTCAATACTGCCTCAG
TCCGACTTGTGTCACCGAGAAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATAACGGAAACAGT
GTTTGGCTCTATTAACGGAATGTTCACTAAGATTATGCCCTTGTCA

1551 TGTAAAGATCCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCAGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTAAATTGTATAGTGGATTGGTAGAT
AGTTCTTACTACCTTGGTAAAATTAAACATATCACCTAACCAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTAAACAAATCATTCTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTGATAGACAGATTACT
ACCACTGGGTTGGTTATACCAATGGTAATAAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTCCTGCCATGAAAATAGATGGCTAAATAAAAAA
GAGAACGTACACACACAGGACGGTACTTTATCTACCGAATTATTTT

1801 GGACATTGTAATTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTAAACATTGACTTCCGTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP270

156/254

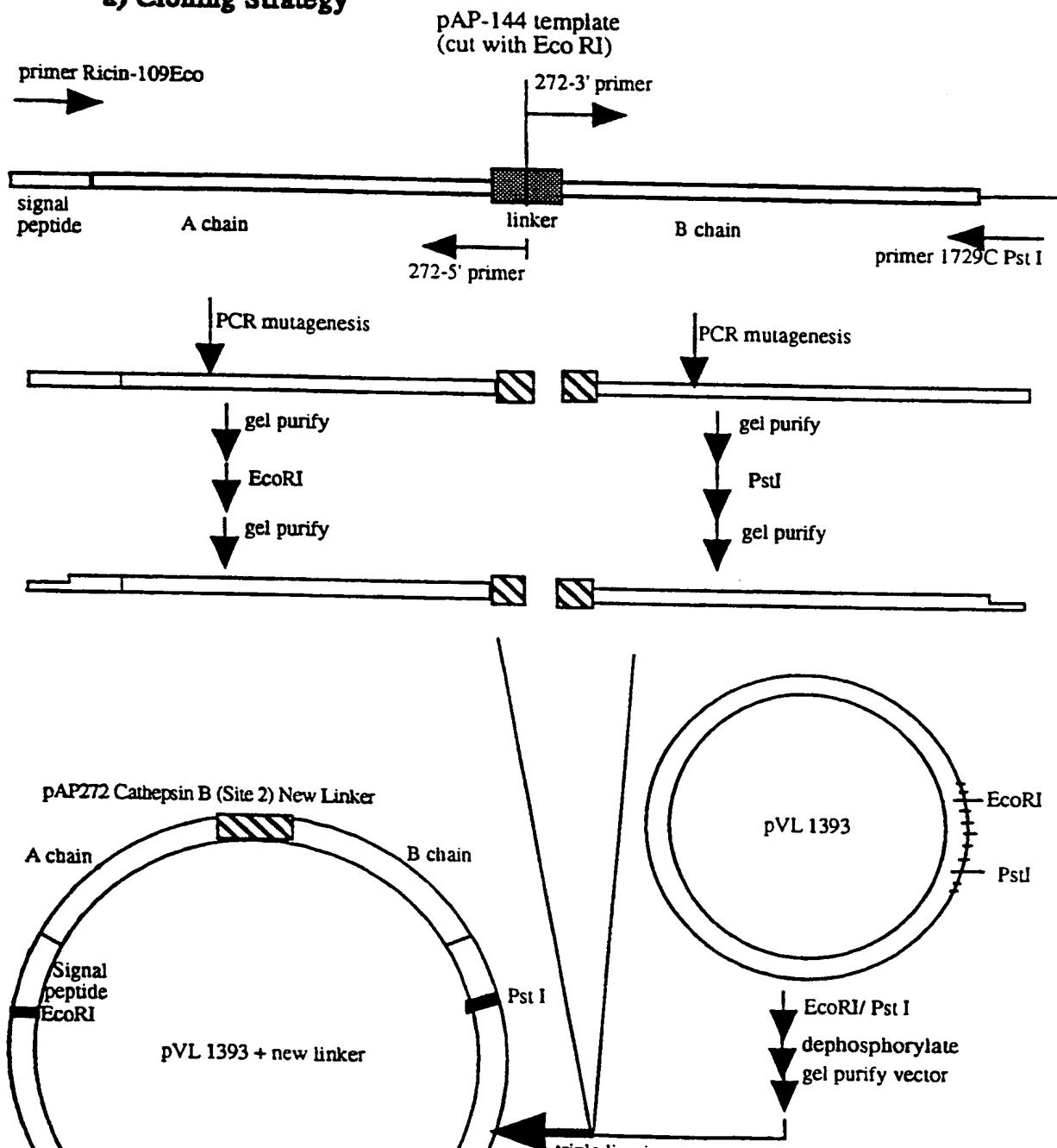
FIGURE 34D

**Amino acid sequence Comparison of Mutant Preproricin Linker
region of MMP-2 to Wild Type**

Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-270 (MMP-2) linker: A chain- S L P L G L W A P N F N -B chain

157/254

FIGURE 35A**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

158/254

FIGURE 35B**Sequence of Cathepsin B (Site 2) Linker Region****WT preprocin linker**

primer 272-3'
5' - AGGATGCCAAATTTAATGCTGATGTTGT - 3'
| * * * *
----- TCTTGCTTATAAGGCCA | GTGGTACCAAATTTAAT -----
----- AGAACGAATATCCGGT | CACCATGGTTAAAATTA -----

3' - AGCAGTGTCAAAAGAAACGAATATCGATCT - 5'
primer 272-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 272 linker**(Cathepsin B Site 2 variant)**

----- TCTTGCTTATAAGCTAGA | AGGATGCCTAATTAAAT -----
----- AGAACGAATATCGATCT | TCCTACGGATTAAAATTA -----

159/254

FIGURE 35C (P1)

Sequence of pAP272 insert

10	20	30	40	50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
 CTTAAGTACTTGGCCCTCCTTATGATAACATTATAACCTACATACGTCA
 51 GGCAACATGGCTTGTGGATCCACCTCAGGGTGGTCTTCACATTAG
 CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAA
 101 AGGATAACAAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
 TCCTATTGTTGTATAAGGGGTTGTTATGGTTAATATTGAAATGGTGT
 151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTGTTGCC
 CGCCCACGGTGACACGTTCGATGTGTTGAAATAGTCTCGACAAGCGCC
 201 TCGTTAACAAACTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT
 251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACCTCA
 TGTCTAACCAAACGGATATTGGTTGCCAATAAAACTCAACTTGAGAGT
 301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
 351 TGTGGTCGGTACCGTGCTGGAAATAGCGCATATTCATCCTGACA
 ACACCAGCCGATGGCACGACCTTATCGCGTATAAGAAAGTAGGACTGT
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTCACTGATGTTCAAAAT
 TAGTCCTTCTACGTCTCGTTAGTAGAGAAAAGTAGACTACAAGTTTA
 451 CGATATACATTGCCTTGGTGGTAATTATGATAGACTTGAACAACTTGC
 GCTATATGTAAGCGAAACCACCATTAATACTATCTGAACCTGTTGAACG
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG
 ACCATTAGACTCTCTTATAGCTAACCTTACCAAGGTGATCTCCTCC
 551 CTATCTAGCGCTTATTACAGTACTGGTGGCACTCAGCTTCCA
 ACTGATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
 601 CTGGCTCGTTCTTATAATTGATCCAAATGATTCAAGCAGCAAG
 GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTCGTT
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
 TAAGGTTATATAACTCCCTCTTACCGCGTGTCTTAATCCATGTTGGCCT

160/254
FIGURE 35C (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAECTCTTATCAACCCCCCTCT
751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTCCTCGGAAACGATCAGGTTA
801 TCAACTGCAAAGACGTAATGGTCCAAATTCAAGTGTACGATGTGAGTA
AGTTGACGTTCTGCATTACCAAGGTTAACGACACATGCTACACTCAT
851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACGCGTGGAGGTGGT
901 TCGTCACAGTTTCTTGCTTATAGCTAGAAGGATGCCATTAAATGC
AGCAGTGTCAAAAGAAAGGAATATCGATCTCCTACGGATTAAAATTACG
951 TGATGTTGTATGGATCCTGAGCCCAGTGCCTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC
1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACACGGAAACGCAATA
CAGATACACAACATACAATCCCTACCTTCTAAGGTGTTGCCCTTGCATTAT
1051 CAGTTGTGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTCAGATTATGTCTACGTTAGTCGAGACCTGAAA
1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAAACTGCTGCA
CCATGTCAGGCCCTCAGATAACACTAGATAACTAACGTTATGACGACGT
1201 ACTGATGCCACCCGCTGGCAAATATGGATAATGGAACCATCATAAATCC
TGACTACGGTGGCGACCCTTATACCTTACCTGGTAGTATTTAGG
1251 CAGATCTAGTCTAGTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTGTCACCATGGTGTG
1301 TTACAGTGCACCAACACATTATGCCGTTAGTCAAGGTTGGCTTCAACT
AATGTCACGTTGGTTGAAATACGGCAATCAGTCCAAACCGAACGGATGA
1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGTAACAACCCGATATACCAGACAC
1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTCGTTATCACCTGTTACCTATCTCCTGACATCGTCACCTT

161/254

FIGURE 35C (P3)

1451 AGGCTGAACAAACAGTGGGCTCTTATGCAGATGGTTCAATACGTCCCTCAG
TCCGACTTGTGTCACCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATAACGGGAAACAGT
GTTTGCGCTCTATTACCGGAATGTTACTAAGATTATGCCCTTGTCA

1551 TGTAAAGATCCTCTCTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCAGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTAAATTGTATAGTGGATTGGTAGAT
AGTTCTTACTACCTGGTAAATTAAACATATCACCTAACCAACATCTA

1651 GTGAGGCGATCGGATCCGAGCCTAAACAAATCATTCTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTGATAGACAGATTACT
ACCACTGGGTTGGTTATACCAATGGTAATAAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGCCTGCCATGAAAATAGATGGCTTAAATAAAAAA
GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTTT

1801 GGACATTGTAATTTGTAAGTGAAGGACAGCAAGTTATCGAATTCC
CCTGTAACATTAAAACATTGACTTCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP272

162/254

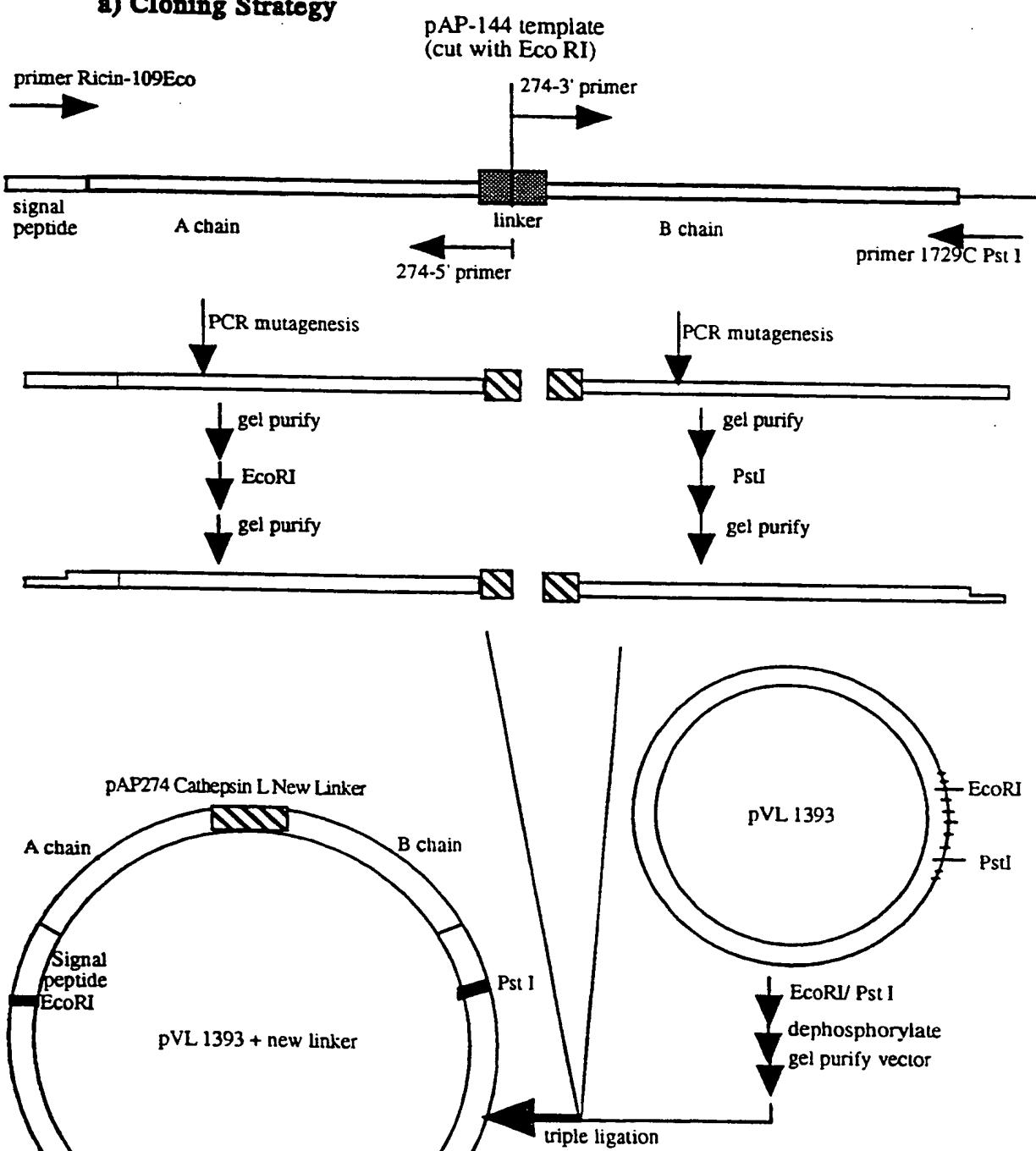
FIGURE 35D

**Amino acid sequence Comparison of Mutant Preproricin Linker
region of Cathepsin B Site 2 to Wild Type**

Wild type rycin linker: A chain- S L L I R P V V P N F N -B chain

pAP-272 (Cathepsin B 2)linker: A chain- S L L I A R R M P N F N -B chain

163/254

FIGURE 36A**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy****SUBSTITUTE SHEET (RULE 26)**

164/254

FIGURE 36B

Sequence of Cathepsin L Linker Region

WT preprocin linker

primer 274-3'
5' - TCATGGGCTAATTTAATGCTGATGTTGT - 3'
| ***** *
----- TCTTGCTTATAAGGCC | GTGGTACCAAATTTAAT -----
----- AGAACGAATATTCCGGT | CACCATGGTTAAAATTA -----
***** *
3' - AGCAGTGTCAAAAGAACGAATATAAGGCC - 5'
primer 274-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 274 linker

(Cathepsin L variant)

----- TCTTGCTTATAATTCCGG | TCATGGGCTAATTTAAT -----
----- AGAACGAATATAAGGCC | AGTACCCGATTAAAATTA -----

165/254

FIGURE 36C (P1)

Sequence of pAP274 insert

10	20	30	40	50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
 CTTAAGTACTTGGCCCTCTTATGATAAACATTATACCTACATACGTCA
 51 GGCAACATGGCTTGTTGGATCCACCTCAGGGTGGTCTTCACATTAG
 CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC
 101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTACCA
 TCCTATTGTTGTATAAGGGTTGTTATGGTTAATATTGAAATGGTGT
 151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTGTCGCC
 CGCCCACGGTGACACGTTCGATGTGTTGAAATAGTCTCGACAAGCGCC
 201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT
 251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACCTCTCA
 TGTCTAACCAACGGATATTGGTTGCCAAATAAAACTAACATTGAGAGT
 301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
 351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTCATCCTGACA
 ACACCAGCCGATGGCACGACCTTATCGCGTATAAGAAAGTAGGACTGT
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTCACTGATGTTAAAAT
 TAGTCCTCTACGTCTCGTTAGTGAGTAGAAAGTGACTACAAGTTTA
 451 CGATATAACATTGCCTTGGTGGTAATTATGATAGACTTGAACAACTTGC
 GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG
 ACCATTAGACTCTTTATAGCTAACCCCTTACCAAGGTGATCTCCTCC
 551 CTATCTCAGCGTTATTACAGTACTGGTGGCACTCAGCTTCCA
 GATAGAGTCGCGAAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
 601 CTGGCTCGTCTTATAATTGCATCCAAATGATTCAGAAGCAGCAAG
 GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTCTTC
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
 TAAGGTTATATAACTCCCTCTTACCGTGCTTTAATCCATGTTGGCCT

166/254

FIGURE 36C (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA
CTAGACGTGGCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTCTCAGATTGGTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTCAAATTCAAGTGTGTACGATGTGAGTA
AGTTGACGTTCTGCATTACCAAGGTTAACGACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTCTTGCTTATATTCCGGTCATGGCTAATTAAATGC
AGCAGTGTCAAAAGAAAGGAATATAAGGCCAGTACCCGATTAAAATTACG

951 TGATGTTGTATGGATCCTGAGCCATAGTGCCTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACATACTACCTACCTTCTAACGGTTGCTTGCCTTGCCTTAT

1051 CAGTTGTGCCATGCAAGTCTAACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTCAAGATTGTCTACGTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTGATCTAACGGAAAGTGTAACTACTTACG
CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAAACTGCTGCA
CCATGTCAGGCCCTCAGATACACTAGATAACTAACGTTATGACGACGT

1201 ACTGATGCCACCGCTGGCAAATATGGATAATGGAACCATCATAAATCC
TGACTACGGTGGCGACCCTTATACCTTACCTGGTAGTATTAGG

1251 CAGATCTAGTCTAGTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTATGCCGTTAGTCAGGTTGGCTTCTACT
AATGTCACGTTGGTTGTAACGCAATCAGTCCAAACCGAAGGATGA

1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTCGTTATCACCTGTTACACCTATCTCCTGACATCGTCACCTT

167/254

FIGURE 36C (P3)

1451 AGGCTGAACAAACAGTGGGCTTTATGCAGATGGTCAATACTGCCTCAG
TCCGACTTGTGTCACCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATAACGGAACAGT
GTTTGCGCTCTATTACCGAATGTTCACTAAGATTATATGCCCTTGTCA

1551 TGTAAAGATCCTCTTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTAAATTGTATAGTGGATTGGTAGAT
AGTTCTTACTACCTTGGTAAATTAAACATATCACCTAACCAATCTA

1651 GTGAGGCATCGGATCCGAGCCTTAAACAAATCATTCTTACCCCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTACCATTTGATAGACAGATTACT
ACCACTGGGTTGGTTATACCAATGGTAATAAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAAA
GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTT

1801 GGACATTGAAATTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTAAACATTGACTTCCGTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP274

168/254

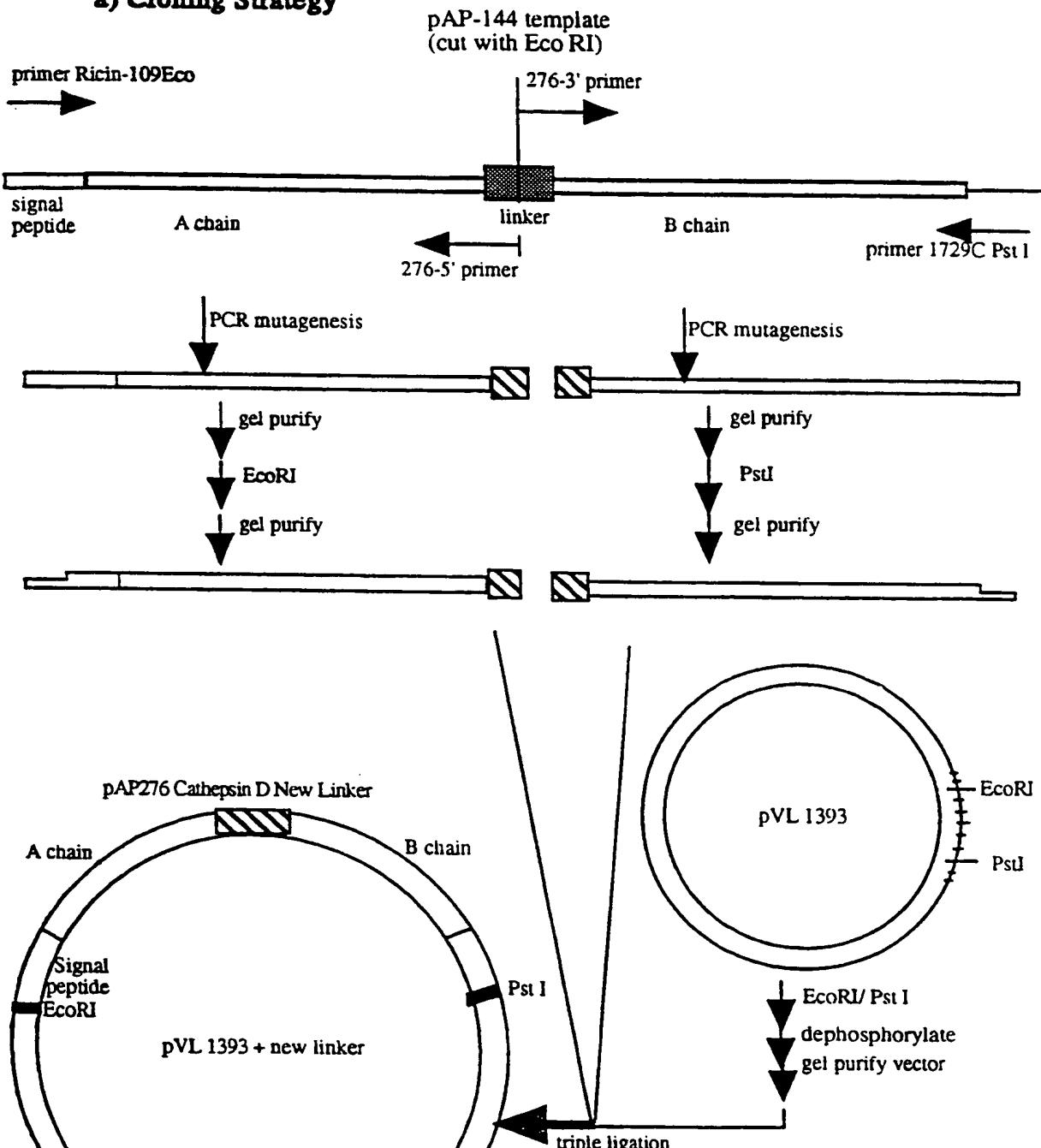
FIGURE 36D

**Amino acid sequence Comparison of Mutant Pre ricin Linker
region of Cathepsin L to Wild Type**

Wild type rycin linker: A chain- S L L I R P V V P N F N -B chain

pAP-274 (Cathepsin L)linker: A chain- S L L I F R S W A N F N -B chain

169/254

FIGURE 37A**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy****SUBSTITUTE SHEET (RULE 26)**

170/254

FIGURE 37B**Sequence of Cathepsin D Linker Region****WT preprocin linker**

primer 276-3'

5' - ACTGTTATTGTTATCACC GCTGATGTTTGT - 3'

| * * * * * * * * * *

----- TCTTTGCTTATAAGGCCA | GTGGTACCAAATTTAAT -----
----- AGATAACGAATATTCCGG | CACCATGGTTAAAATTA -----

* * * * * * * * *

3' - AGCAGTGTCAAAAGACCACAACAGTAGCGA - 5'

primer 276-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 276 linker**(Cathepsin D variant)**----- TCTGGTGTTGTCATCGCT | ACTGTTATTGTTATCACC -----
----- AGACCACAAACAGTAGCGA | TGACAATAACAATAGTGG -----

171/254

FIGURE 37C (P1)

Sequence of pAP276 insert

10	20	30	40	50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
 CTTAAGTACTTGCCCTCCTTATGATAACATTATACCTACATACGTCA
 51 GGCAACATGGCTTGTTGGATCCACCTCAGGGTGGTCTTCACATTAG
 CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC
 101 AGGATAACAAACATATTCCCCAAACAATACCCAATTATAAACTTACCA
 TCCTATTGTTGTATAAGGGTTGTTATGGGTTAATATTTGAAATGGTGT
 151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTGTTGCCGG
 CGCCCACGGTGACACGTTGATGTGTTGAAATAGTCTGACAAGCGCC
 201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAAGTGTGCCAA
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT
 251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACCTCTCA
 TGTCTAACCAACGGATATGGTTGCCAAATAAAACTAACATTGAGAGT
 301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
 351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTCTTCATCCTGACA
 ACACCAGCCGATGGCACGACCTTATCGCGTATAAGAAAGTAGGACTGT
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTCACTGATGTTCAAAAT
 TAGTCCTTCTACGTCTCGTTAGTGGAGTAGAAAAGTGAACACTACAAGTTTA
 451 CGATATACATTGCCCTTGGTGGTAATTATGATAGACTTGAACAACTTGC
 GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG
 ACCATTAGACTCTCTTATAGCTCAACCCTTACCAAGGTGATCTCCTCC
 551 CTATCTCAGCGCTTATTACAGTACTGGTGGCACTCAGCTTCCA
 GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
 601 CTGGCTCGTTCTTATAATTGCATCCAAATGATTCAAGCAGCAAG
 GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTCGTT
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
 TAAGGTTATATAACTCCCTCTTACCGCGTGTCTTAATCCATGTTGGCCT

172/254

FIGURE 37C (P2)

701 GATCTGCAACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA
CTAGACGTGGCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTCTCAGATTGGTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTCAAATTCACTGAGTGTACGATGTGAGTA
AGTTGACGTTCTGCATTACCAAGGTTAACGACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTCTGGTGTTCATCGCTACTGTTATTGTTATCACCAG
AGCAGTGTCAAAAGACCACAACAGTAGCGATGACAATAACAATAGTGGCG

951 TGATGTTGTATGGATCCTGAGCCCAGTGCGTATCGTAGGTCGAAATG
ACTACAAACATACTACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATAACACAACATACTACCTACCTCTAACGGTTAGGTGTCCTTGCCTTAT

1051 CAGTTGTGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTAGATTATGTCTACGTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTACG
CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATAACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGATAATGGAACCATCATAAATCC
TGACTACGGTGGCGACCCTTATACCTTACCTTGGTAGTATTAGG

1251 CAGATCTAGTCTAGTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTATGCCGTTAGTCAAGGTTGGCTTCTACT
AATGTCACGTTGGTTGAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATAACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTAAA
GAACGTTGTTATCACCTGTTACACCTATCTCCTGACATCGTCACTTT

173/254

FIGURE 37C (P3)

1451 AGGCTGAACAAACAGTGGGCTTTATGCAGATGGTTCAATACGTCCCTCAG
TCCGACTTGTGTCACCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGAAACAGT
GTTTGGCTCTATTACCGGAATGTTACTAAGATTATATGCCCTTGTCA

1551 TGTAAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTAAATTGTATAGTGGATTGGTAGAT
AGTTCTTACTACCTTGGTAAATTAAACATATCACCTAACCAACATCTA

1651 GTGAGGCGATCGGATCCGAGCCTAAACAAATCATTCTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTGATAGACAGATTACT
ACCACTGGGTTGGTTATACCAATGGTAATAAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTCTGCCATGAAAATAGATGGCTAAATAAAAAA
GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTTT

1801 GGACATTGTAATTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTAAACATTGACTTCCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP276

174/254

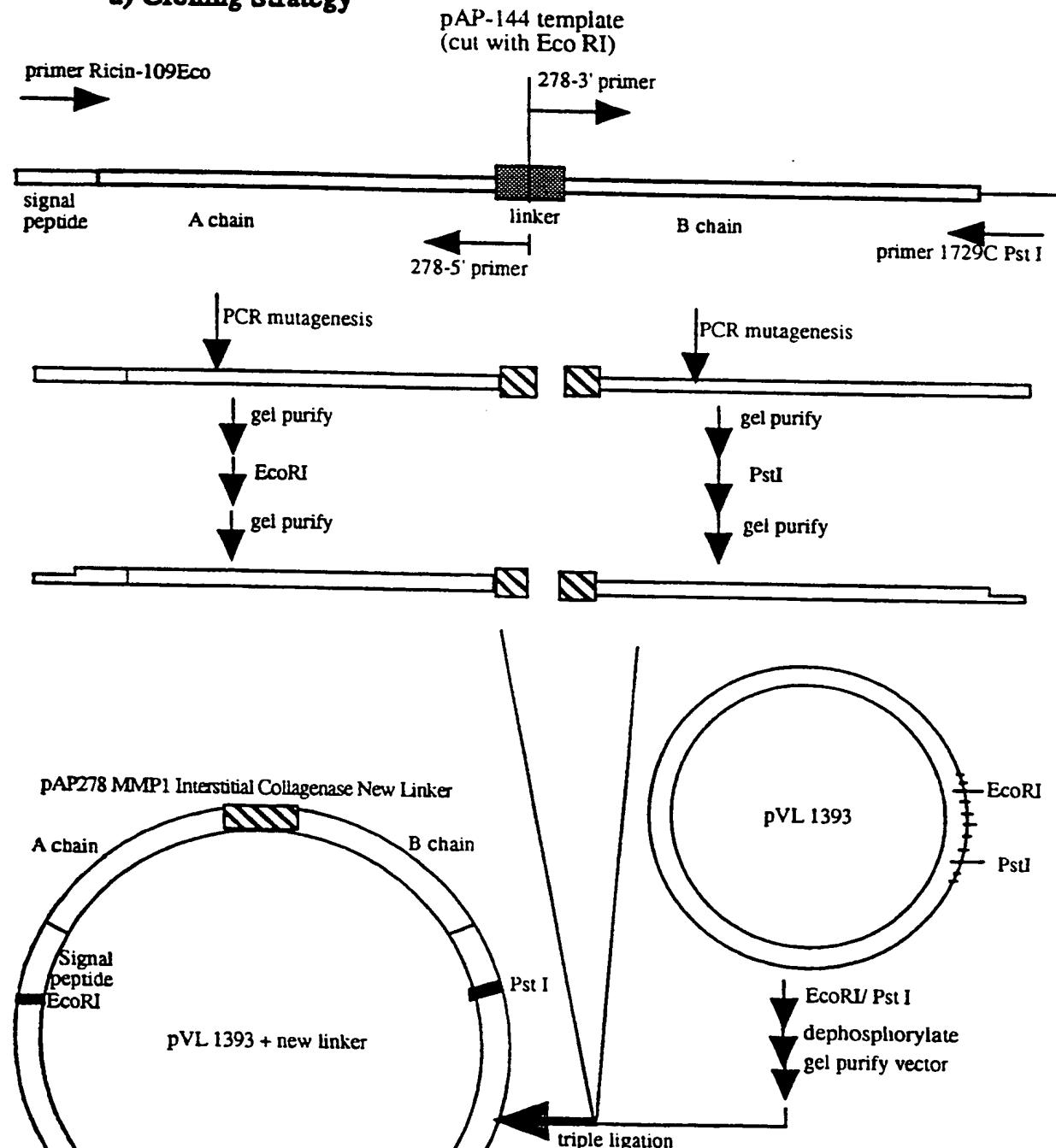
FIGURE 37D

**Amino acid sequence Comparison of Mutant Preproricin Linker
region of Cathepsin D to Wild Type**

Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-276 (Cathepsin D) linker: A chain- S G V V I A T V I V I T -B chain

175/254

FIGURE 38A**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

176/254

FIGURE 38B**Sequence of MMP-1 (Interstitial collagenase) Linker Region****WT preprocin linker**

primer 278-3'
5' - ATTTGGGGACAGTTAACGCTGATGTTGT - 3'
* * * * * * * *
----- TCTTGCTTATAAGGCCA | GTGGTACCAAATTAAAT -----
----- AGAAACGAATATTCCGGT | CACCATGGTTAAAATTA -----
* * * * * * * *
3' - AGCAGTGTCAAAAGAAACCCAGGAGTTCCG - 5'
primer 278-5'

1) PCR mutagenesis

2) Ligate with pVL1393

**pAP 278 linker
(MMP-1 variant)**

----- TCTTGCGGTCTCAAGGC | ATTTGGGGACAGTTAACGCTGATGTTGT -----
----- AGAAACCCAGGAGTTCCG | TAAACCCCTGTCAAATTA -----

177/254

FIGURE 38C (P1)

Sequence of pAP278 insert

1	10 GAATT	20 CATGAAACCGGGAGGAAATACTATT	30 GTAATATGGATGTATGCAGT	40 CTTAAGTACTTGGCCCTCCTTATGATAACATTACACATACGTCA	50
51	GGCAACATGGCTTGTTGGATCCACCTCAGGGTGGTCTTCACATTAG CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAA				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA TCCTATTGTTGTATAAGGGGTTGTTATGGGTTAATATTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTGTTGCCG CGCCCACGGTGACACGTTCGATGTGTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGGTGC AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT				
251	ACAGAGTTGGTTGCCCTATAAACCAACGGTTATTTAGTTGA ACTCTCAACCAAACGGATATTGGTGC AAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTC ACCAATGCATA TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTCTTC ATCCTGACA ACACCAGCCGATGGCACGACCTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTCA CTGATGTTCAAAAT TAGTCCTCTACGTCTCGTTAGTGAGTAGAAAAGTGA CTACAAGTTA				
451	CGATATA CATTCGCCTTGGGTAATTATGATAGACTTG AACAACTTGC GCTATATGTAAGCGGAAACCACCA TTAAACTATCTGA ACTTGTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCC ACTAGAGGAGG ACCATTAGACTCTCTTTATAGCTCA ACCCTTACCA GGTGATCTCCTCC				
551	CTATCTCAGCGCTTATTACAGTACTGGTGGCACTCAGCT CCA ACT GATAGAGTC CGAAATAATA ATGTC ATGACCACCGT GAGTC GAAGGTTGA				
601	CTGGCTCGTCTTATAATTGC ATCCAAATGATT TCAGAAGCAGCAAG GACCGAGCAAGGAAATATTAA ACGTAGGTTACTAAAGTCTCGTC GTTC				
651	ATTCCA ATATATTGAGGGAGAAATGCG CACGAGAATTAGGT ACAACCGGA TAAGGTT ATATACTCC CTTACCG CGTCTTA ATCC ATGTTGGC CT				

178/254

FIGURE 38C (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA
 CTAGACGTGGCTAGGATCGCATTAAATGTGAACCTTATCAACCCCTCT

 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT
 GAAAGGTGACGTTAAGTTCTCAGATTGGTCCTCGAAACGATCAGGTTA

 801 TCAACTGCAAAGACGTAATGGTCAAATTCAAGTGTACGATGTGAGTA
 AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

 851 TATTAATCCCTATCATAGCTCTCATGGTGTAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACGCGTGGAGGTGGT

 901 TCGTCACAGTTCTTGCGCCTCAAGGCATTGGGACAGTTAATGC
 AGCAGTGTCAAAAGAAACGCAGGAGTCCGTAACCCCTGTCAAATTACG

 951 TGATGTTGTATGGATCCTGAGCCATAGTGCATCGTAGGTCGAAATG
 ACTACAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC

 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATAACACAACATACTACCCCTACCTCTAAGGTGTTGCCTTGCCTTAT

 1051 CAGTTGTGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTCAAGATTGTCTACGTTAGTCGAGACCTGAAA

 1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC

 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAAACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTAGATAACTAACGTTATGACGACGT

 1201 ACTGATGCCACCGCTGGCAAATATGGATAATGGAACCACATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCTATTACCTGGTAGTATTAGG

 1251 CAGATCTAGTCTAGTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTGTCACCATGGTGTG

 1301 TTACAGTGCAAACCAACATTATGCCGTTAGTCAGGTTGGCTTCTACT
 AATGTCACGTTGGTTGTAACACGGCAATCAGTCCAACCGAAGGATGA

 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTGTTATCACCTGTTACACCTATCTCCTGACATCGTCACCTT

179/254

FIGURE 38C (P3)

1451 AGGCTGAACAACAGTGGGCTTTATGCAGATGGTCATAACGTCCCTCAG
TCCGACTTGTGTCACCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATAACGGAAACAGT
GTTTGGCTCTATTAACGGAATGTTCACTAAGATTATGCCCTTGTCA

1551 TGTAAAGATCCTCTTGTCGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCAGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTAAATTGTATAGTGGATTGGTAGAT
AGTTCTTACTACCTGGTAAAATTAAACATATCACCTAACCAACATCTA

1651 GTGAGGCGATCGGATCCGAGCCTAAACAAATCATTCTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTACCATTTGATAGACAGATTACT
ACCACTGGTTGGTTATACCAATGGTAATAAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGCCTGCCATGAAAATAGATGGCTAAATAAAAAA
GAGAACGTCACACACAGGACGGTACTTTATCTACCGAATTATTTT

1801 GGACATTGTAATTTGTAAGTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTAAACATTGACTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP278

180/254

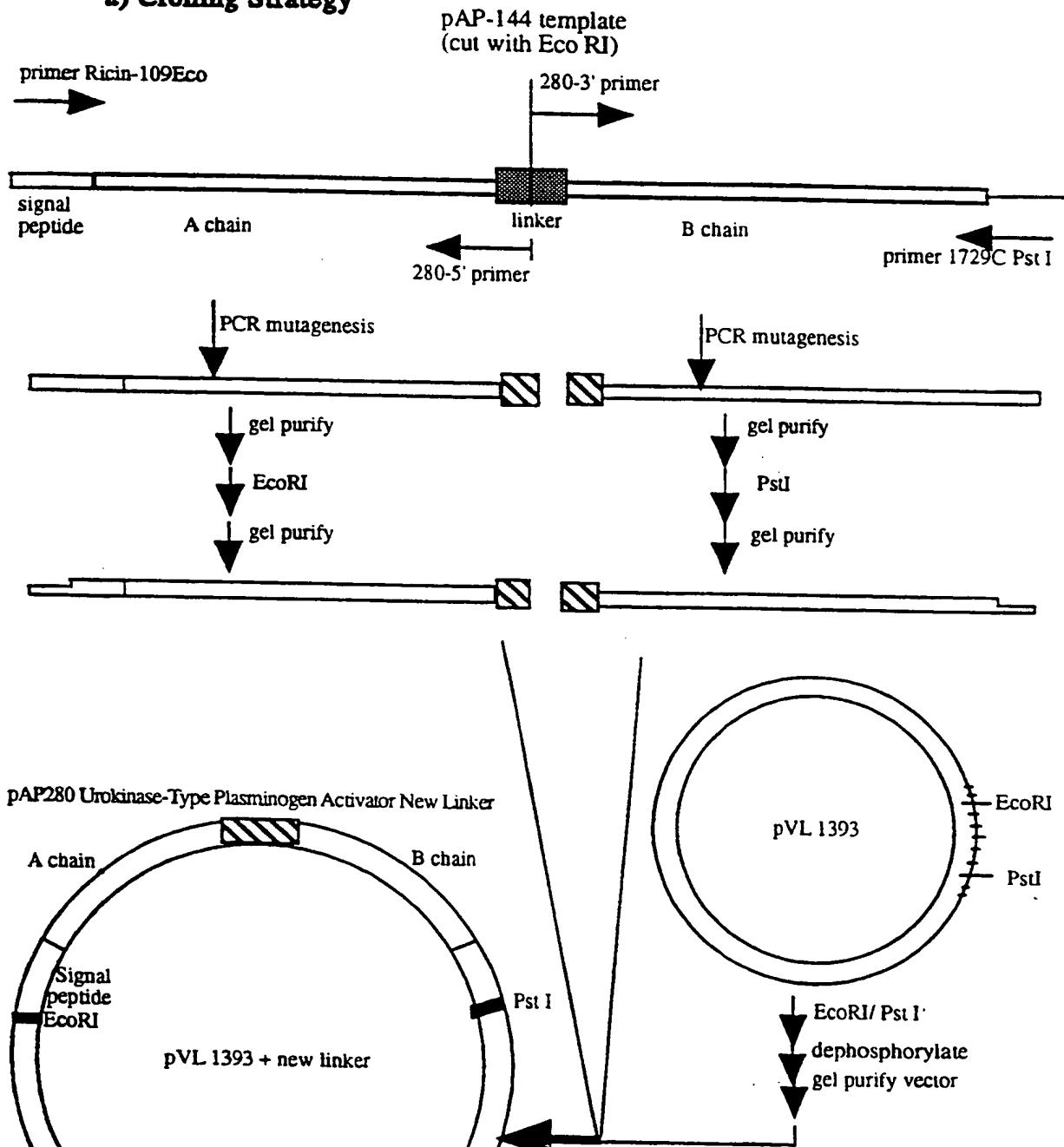
FIGURE 38D

Figure 38. d) Amino acid sequence Comparison of Mutant Preproricin Linker region of MMP-1 (Interstitial collagenase) to Wild Type

Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-278 (MMP-1) linker: A chain- S L G P Q G I W G Q F N -B chain

181/254

FIGURE 39A**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

182/254

FIGURE 39B**Sequence of Urokinase-Type Plasminogen Activator Linker Region****WT preprocin linker**

primer 280-3'

5' - GTTGTGGTGGCTCTGTAGCTGATGTTGT - 3'
* * * * * * *-----
----- TCTTGCTTATAAGGCCA | GTGGTACCAAATTTAAT -----
----- AGAAACGAATATTCCGGT | CACCATGGTTAAAATTA -----
* * * * * * * * *

3' - AGCAGTGTCAAATTTTAGGGGACCTTCT - 5'

primer 280-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 280 linker

(uPA variant)

----- AAAAAATCCCCTGGAAGA | GTTGTGGTGGCTCTGTA -----
----- TTTTTTAGGGGACCTTCT | CAACAGCCACCGAGACAT -----

183/254

FIGURE 39C (P1)

Sequence of pAP280 insert

1	10	20	30	40	50
---	----	----	----	----	----

GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
 CTTAAGTACTTGGCCCTCCTTATGATAACATTATACTACATACGTCA
 GGCAACATGGCTTGTGATCCACCTCAGGGTGGTCTTCACATTAG
 CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAA
 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCA
 TCCTATTGTTGTATAAGGGTTGTTATGGGTTAATATTTGAAATGGTGT
 GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTGCC
 CGCCCACGGTGACACGTTGATGTGTTGAAATAGTCTCGACAAGCGCC
 TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTGCC
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGT
 ACAGAGTTGGTTGCCTATAAAACCAACGGTTATTTAGTTGAACCTCTCA
 TGTCACCAACAAACGGATATTGGTTGCCAAATAAAATCAACTTGAGAGT
 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTCTTCACTCGCGTATA
 ACACCAGCCGATGGCACGACCTTATCGCGTATAAGAAAGTAGGACTGT
 ATCAGGAAGATGCAGAAGCAATCACTCATTTTCACTGATGTTCAAAAT
 TAGTCCTCTACGTCTCGTTAGTGAGTAGAAAAGTGAACAGTTTA
 CGATATACATTGCCCTTGGTGGTAATTATGATAGACTGAAACAACCTGC
 GCTATATGTAAGCGGAAACCAACCATTAATAACTATCTGAACCTGTTGAACG
 TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG
 ACCATTAGACTCTTTATAGCTCAACCCTTACCACTGGTGTCTCCTCC
 CTATCTAGCGCTTATTATTACAGTACTGGTGGCACTCAGCTTCCA
 GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
 CTGGCTCGTTCTTATAATTGCATCCAAATGATTCAGAAGCAGCAAG
 GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTCGTT
 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
 TAAGGTTATATAACTCCCTTTACCGTGCTTAATCCATGTTGGCCT

184/254

FIGURE 39C (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA
 CTAGACGTGGCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT
 GAAAGGTGACGTTAAGTCTCAGATTGGTCCTCGGAAACGATCAGGTTA
 801 TCAAATGCAAAGACGTAATGGTCAAATTCAAGTGTACGATGTGAGTA
 AGTTGACGTTCTGCATTACCAAGGTTAACGACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACGCGTGGAGGTGGT
 901 TCGTCACAGTTAAAAAAATCCCCTGGAAGAGTTGTGGCTCTGTAGC
 AGCAGTGTCAAATTAGGGACCTCTCAACAGCCACCGAGACATCG
 951 TGATGTTGTATGGATCCTGAGCCCATACTGCGTATCGTAGGTGAAATG
 ACTACAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATAACACAACATACTACAAATCCCTACCTTCTAAGGTGTTGCCTTGCCTTAT
 1051 CAGTTGTGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTCAAGATTATGTCTACGTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAAAACTGCTGCA
 CCATGTCAGGCCCTCAGATAACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCGCTGGCAAATATGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCTTACCTTGGTAGTATTAGG
 1251 CAGATCTAGTCTAGTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTGTCACCATGGTGTG
 1301 TTACAGTGCACCAACACATTATGCCGTTAGTCAGGTTGGCTTCAAGGTTGGCTTCTACT
 AATGTCACGTTGGTTGTAATACGGCAATCAGTTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACAATGTTGTAACAACCGATATAACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTCGTTATCACCTGTTACCTATCTCCTGACATCGTCACTT

185/254

FIGURE 39C (P3)

1451 AGGCTGAACAAACAGTGGGCTTTATGCAGATGGTCAATAACGTCCCTCAG
TCCGACTTGTGTCACCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATAACGGGAAACAGT
GTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTGTCA

1551 TGTAAAGATCCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACC GGTTGCTACCTACAA

1601 TCAAGAACATGATGGAACCATTAAATTGTATAGTGGATTGGTAGAT
AGTTCTTACTACCTGGTAAAATTAAACATATCACCTAACCAACAACTCA

1651 GTGAGGCGATCGGATCCGAGCCTAAACAAATCATTCTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTGATAGACAGATTACT
ACCACTGGGTTGGTTATACCAATGGAATAAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGCCTGCCATGAAAATAGATGGCTTAAATAAAAAA
GAGAACGTACACACACAGGACGGTACTTTATCTACCGAATTATTTT

1801 GGACATTGTAATTTGTAAGTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTCCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

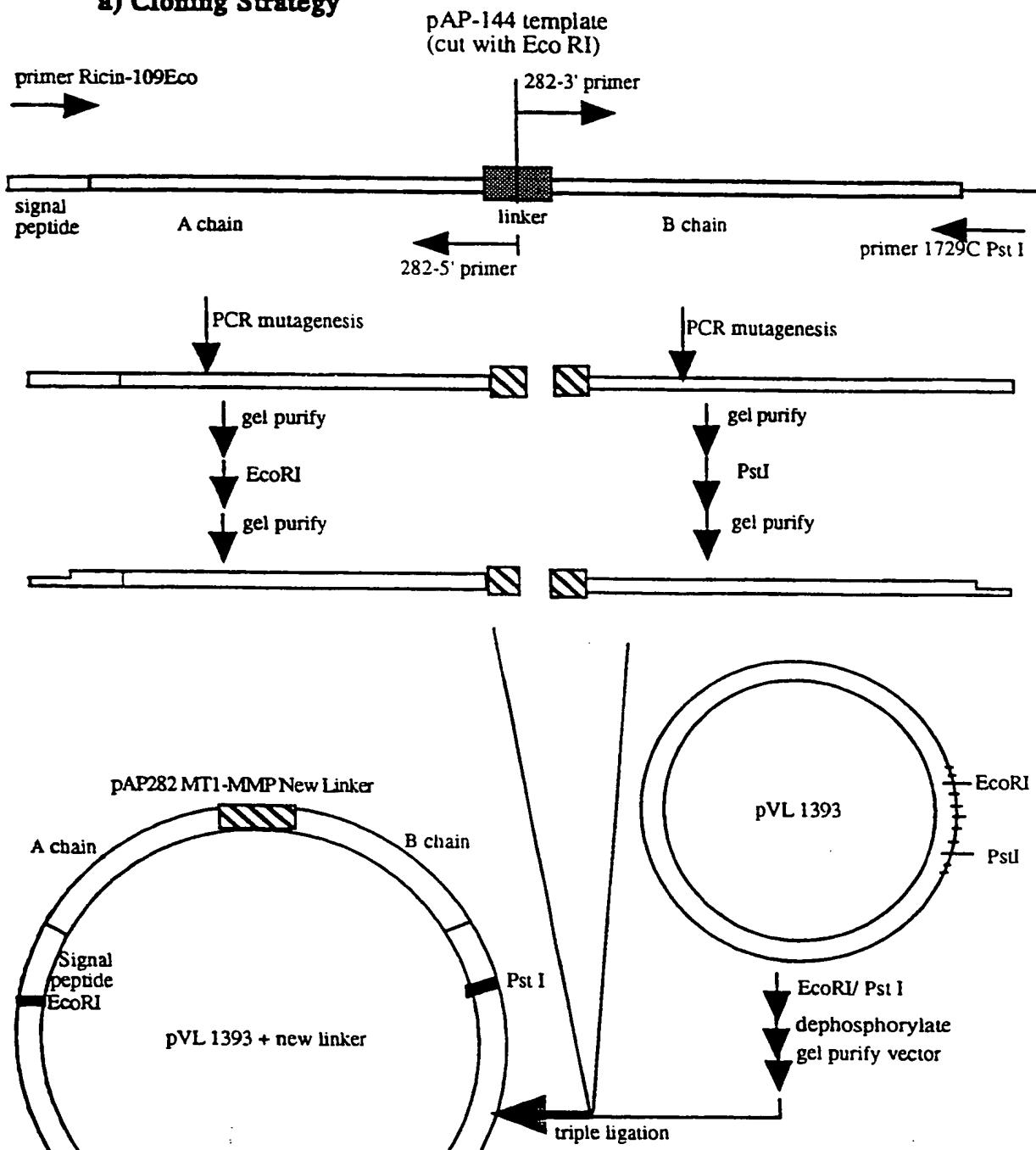
Sequence name: PAP280

186/254

FIGURE 39D**Figure 39. d) Amino acid sequence Comparison of Mutant Preprorcin Linker region of Urokinase-Type Plasminogen Activator to Wild Type**

Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain
pAP-280 (uPA) linker: A chain- K K S P G R V V G G S V-B chain

187/254

FIGURE 40A**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy****SUBSTITUTE SHEET (RULE 26)**

188/254

FIGURE 40B**Sequence of MT-MMP Linker Region****WT preprocin linker**

primer 282-3'
5' - GCTCCTGGTATTCTGGCGCTGATGTTGT - 3'

----- TCTTGCTTATAAGGCCA | GTGGTACCAAATTTAAT -----
----- AGAAACGAATATTCCGGT | CACCATGGTTAAAATTA -----
----- * * * * * -----
3' - AGCAGTGTCAAAGGGGTTCCCTGAGGATCCC - 5'
primer 282-5'

1) PCR mutagenesis

2) Ligate with pVL1393

**pAP 282 linker
(MT-MMP variant)**

----- CCCCAAGGACTCCTAGGG | GCTCCTGGTATTCTGGC -----
----- GGGGTTCCCTGAGGATCCC | CGAGGGACCATAAGAACCG -----

189/254

FIGURE 40C (P1)

Sequence of pAP282 insert

1	10 	20 	30 	40 	50
---	--------	--------	--------	--------	--------

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
 CTTAAGTACTTGGCCCTCCTTATGATAACATTATACTACACATACGTCA
 51 GGCAACATGGCTTGTTGGATCCACCTCAGGGTGGCTTACATTAG
 CCGTTGTACCGAAAACACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC
 101 AGGATAACAACATATTCCCCAACAAATACCAATTATAAACTTTACCA
 TCCTATTGTTGATAAGGGTTATGGGTTAATATTGAAATGGTGT
 151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTGTTGCCGG
 CGCCCACGGTGACACGTTGATGTGTTGAAATAGTCTCGACAAGCGCC
 201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT
 251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACCTCTCA
 TGTCTCAACCAAACGGATATGGTTGCCAAATAAAATCAACTTGAGAGT
 301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTACCAATGCATA
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
 351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTCATCCTGACA
 ACACCAGCCGATGGCACGACCTTATCGCGTATAAGAAAGTAGGACTGT
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTCACTGATGTTAAAAT
 TAGTCCTCTACGTCTCGTTAGTAGAAAGTAGACTACAAGTTTA
 451 CGATATAACATTGCCTTGGTGGTAATTATGATAGACTTGAACAACTTGC
 GCTATAATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG
 ACCATTAGACTCTCTTATAGCTAACCCCTTACCAAGGTGATCTCCTCC
 551 CTATCTCAGCGCTTATTATTACAGTACTGGTGGCACTCAGCTTCCA
 ACTGATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
 601 CTGGCTCGTCTTATAATTGCATCAAATGATTCAAGCAGCAAG
 GACCGAGCAAGGAAATATTAACGTAGGTTACTAAAGTCTCGTGTTC
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
 TAAGGTTATATAACTCCCTCTTACCGTGTCTTAATCCATGTTGGCCT

190/254

FIGURE 40C (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA
 CTAGACGTGGTCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT
 GAAAGGTGACGTTAAGTTCTCAGATTGGTCCTCGGAAACGATCAGGTTA
 801 TCAACTGCAAAGACGTAATGGTCAAATTCAAGGTTAACGATGTGAGTA
 AGTTGACGTTCTGCATTACCAAGGTTAACGTACACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACGCGTGGAGGTGGT
 901 TCGTCACAGTTCCCCAAGGACTCCTAGGGCTCCTGGTATTCTGGCGC
 AGCAGTGTCAAAGGGTCTGAGGATCCCCGAGGACCATAAGAACCGCG
 951 TGATGTTGTATGGATCCTGAGCCCAGTGCATCGTAGGTGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACCAATCCCTACCTTCTAAGGTGTTGCCTTGCCTTAT
 1051 CAGTTGTGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTAGATTATGTCAGTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAAAACTGCTGCA
 CCATGTCAGGCCCTCAGATAACACTAGATAACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCACATCAAATCC
 TGACTACGGTGGCGACCGTTATACCCATTACCTGGTAGTATTAGG
 1251 CAGATCTAGTCTAGTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAATCGCTGTAGTCCCTGTCACCATGGTGTG
 1301 TTACAGTCAAACCAACATTATGCCGTTAGTCAGGTTGGCTTCTACT
 AATGTCACGTTGGTTGAAATACGGCAATCAGTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTGTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATAACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAA
 GAACGTTCGTTATCACCTGTTACACCTATCTCCTGACATCGTCACTTT

191/254

FIGURE 40C (P3)

1451 AGGCTGAACAAACAGTGGGCTCTTATGCAGATGGTCATAACGTCCAG
TCCGACTTGTTGTCACCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATAACGGGAAACAGT
GTTTGCTCTATTACCGAATGTTCACTAAGATTATATGCCCTTGTCA

1551 TGTTAAGATCCTCTCTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTAAATTGTATAGTGGATTGGTGTAGAT
AGTTCTTACTACCTGGTAAAATTAAACATATCACCTAACCAACATCTA

1651 GTGAGGCGATCGGATCCGAGCCTAAACAAATCATTCTTACCCCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTTGATAGACAGATTACT
ACCACTGGGTTGGTTATACCAATGGTAAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGCCTGCCATGAAAATAGATGGCTTAAATAAAAAA
GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTT

1801 GGACATTGTAATTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTAAAACATTGACTTCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

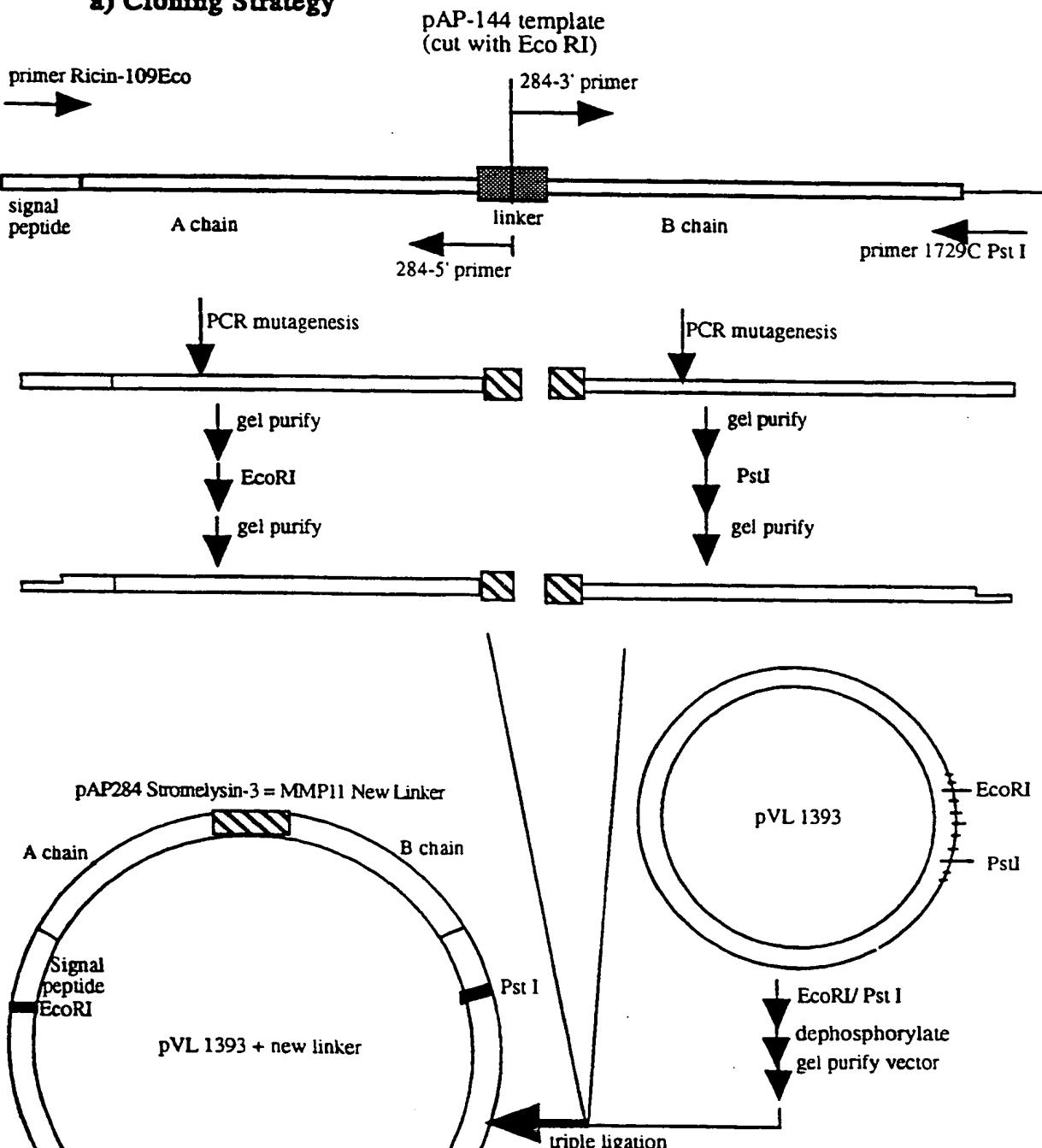
Sequence name: PAP282

192/254

FIGURE 40D**Amino acid sequence Comparison of Mutant Preproricin Linker
region of MT-MMP to Wild Type**

Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain
pAP-282 (MT-MMP) linker: A chain- P Q G L L G A P G I L G-B chain

193/254

FIGURE 41A**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy****SUBSTITUTE SHEET (RULE 26)**

194 / 254

FIGURE 41B**Sequence of MMP-11 (Stromelysin-3) Linker Region****WT preprocin linker**

primer 284 - 3'

5' - ATGGAAAGGCCATGCTCGTTAGTTCATGTCAGAGGCCACACTGGCTATGGATGTTGTATGCA 3'
 |
 ... - TCTTTGCTTATAAGGCCA | GTGGTACCAAATTTTAAAT ...
 ... - AGAAACGAAATTCCGGT | CACCATGGTTAAATTAA ...
 ... -
 3' - GGTTGGTAGCAGTGTCAAAAGTGCCTGGGCTCCCAAAATTCTCACCCCTAAATACTTAGCTGCAAG - 5'
 primer 284 - 5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 284 linker

(MMP-11 variant)

... - CACGGCCCCGAGGGTTAACAGTGGGATTTTATGAATCTGACGTC | ATGGAAAGAGGCCATGCTCGTTAGTTCATGTCAGAGGCCACACT ...
 ... - GTGCCGGGGCTCCCAAAATTCTCACCCCTAAATACTTAGCTGCAAG | TACCCCTCTCCGGTACCGGAAATCAAGTACAGCAACTCGGAAGTGTGA ...

195/254

FIGURE 41C (P1)

Sequence of pAP284 insert

10	20	30	40	50

1 GAATTCATGAAACCGGGAGGAATACTATTGTAATATGGATGTATGCAGT
 CTTAAGTACTTGGCCCTCCTTATGATAACATTATACCTACATACGTCA
 51 GGCAACATGGCTTGTGATCCACCTCAGGGTGGTCTTCACATTAG
 CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC
 101 AGGATAACAAACATATTCCCCAAACAATACCCAATTATAAACCTTACCA
 TCCTATTGTTGTATAAGGGTTGTTATGGGTTAATATTGAAATGGTGT
 151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTGTCGCC
 CGCCCACGGTGACACGTTCGATGTGTTGAAATAGTCTCGACAAGCGCC
 201 TCGTTAACAACTGGAGCTGATGTGAGACATGATACCAAGTGTGCCAA
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT
 251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACCTCTCA
 TGTCTCAACCAAACGGATATTGGTTGCCAAATAAAATCAACTTGAGAGT
 301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTACCAATGCATA
 TTAGTACGTCTCGAAAGACAATGTAATCGCACCTACAGTGGTTACGTAT
 351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTCTTCATCCTGACA
 ACACCAGCCGATGGCACGACCTTATCGGTATAAAAGAAAGTAGGACTGT
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTCACTGATGTTAAAAT
 TAGTCCTTCTACGTCTCGTTAGTGAGTAGAAAGTGAACAGTTTA
 451 CGATATAACATCGCCTTGGTGGTAATTATGATAGACTTGAACAACTTGC
 GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG
 ACCATTAGACTCTTTATAGCTCAACCCTTACCAGGTGATCTCCTCC
 551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTCCA
 ACTGATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
 601 CTGGCTCGTCTTATAATTGCATCAAATGATTTCAGAAGCAGCAAG
 GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTGTTC
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
 TAAGGTTATATAACTCCCTTTACCGTGTCTTAATCCATGTTGGCCT

196/254

FIGURE 41C (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA
CTAGACGTGGTAGGATCGCATTAATGTGAACCTTATCAACCCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTCCAAATTCAAGTGTGACGATGTGAGTA
AGTGACGTTCTGCATTACCAAGGTTAACGTACACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTT
AGCAGTGTCAAA

Linker Sequence:

CACGGCCCCGAGGGTTAACAGAGTGGGATTTATGAATCTGACGTCAATGGG
GTGCCGGGCTCCCAAATTCTCACCTAAAATACCTAGTACGTACGACTACCC

AAGAGGCCATGCTCGTTAGTTATGTCGAAGAGCCTCACACT
TTCTCCGGTACGAGCAAATCAAGTACAGCAACTCGGAGTGTGA

949 GC
CG

951 TGATGTTGATGGATCCTGAGCCCAGTGCCTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC

1001 GTCTATGTGTTGATGGATGGAAAGATTCCACAACGGAAACGCAATA
CAGATACACAACATACCTACCTCTAAGGTGTTGCCTTGCCTTAT

1051 CAGTTGTGCCATGCAAGTCTAACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTAGATTATGTCTACGTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTGATCTAACGAAAGTGTAACTACTTACG
CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC

1151 GGTACAGTCGGGAGTCTATGTGATGATCTATGATTGCAAACTGCTGCA
CCATGTCAGGCCCTCAGATACACTAGATAACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGCGACCGTTATACCTTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTGTCACCATGGTGTG

197/254

FIGURE 41C (P3)

1301 TTACAGTGAAACCAACATTATGCCGTTAGTCAGGGTGGCTTCTACT
AATGTCACGTTGGTTGTAATAACGGCAATCAGTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGTAACAACCCGATATAACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTCGTTATCACCTGTTCATACCTATCTCCTGACATCGTCACCTT

1451 AGGCTGAACAAACAGTGGGCTCTTATGCAGATGGTTCAATACGTCCCTCAG
TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATAACGGGAAACAGT
GTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTGTCA

1551 TGTAAAGATCCTCTCTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTAAATTGTATAGTGGATTGGTGTAGAT
AGTTCTTACTACCTGGTAAATTAACATATCACCTAACCAACATCTA

1651 GTGAGGCGATCGGATCCGAGCCTAAACAAATCATTCTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT

1701 TGGTACCCAAACCAAATATGGTACCATTATTTGATAGACAGATTACT
ACCACTGGGTTGGTTATACCAATGGAATAAAACTATCTGCTAATGA

1751 CTCTTGCACTGTGTGTCTGCCATGAAAATAGATGGCTAAATAAAAAA
GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTT

1801 GGACATTGTAATTTGTAAGTGAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTAAAACATTGACTTCCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

198/254

FIGURE 41D

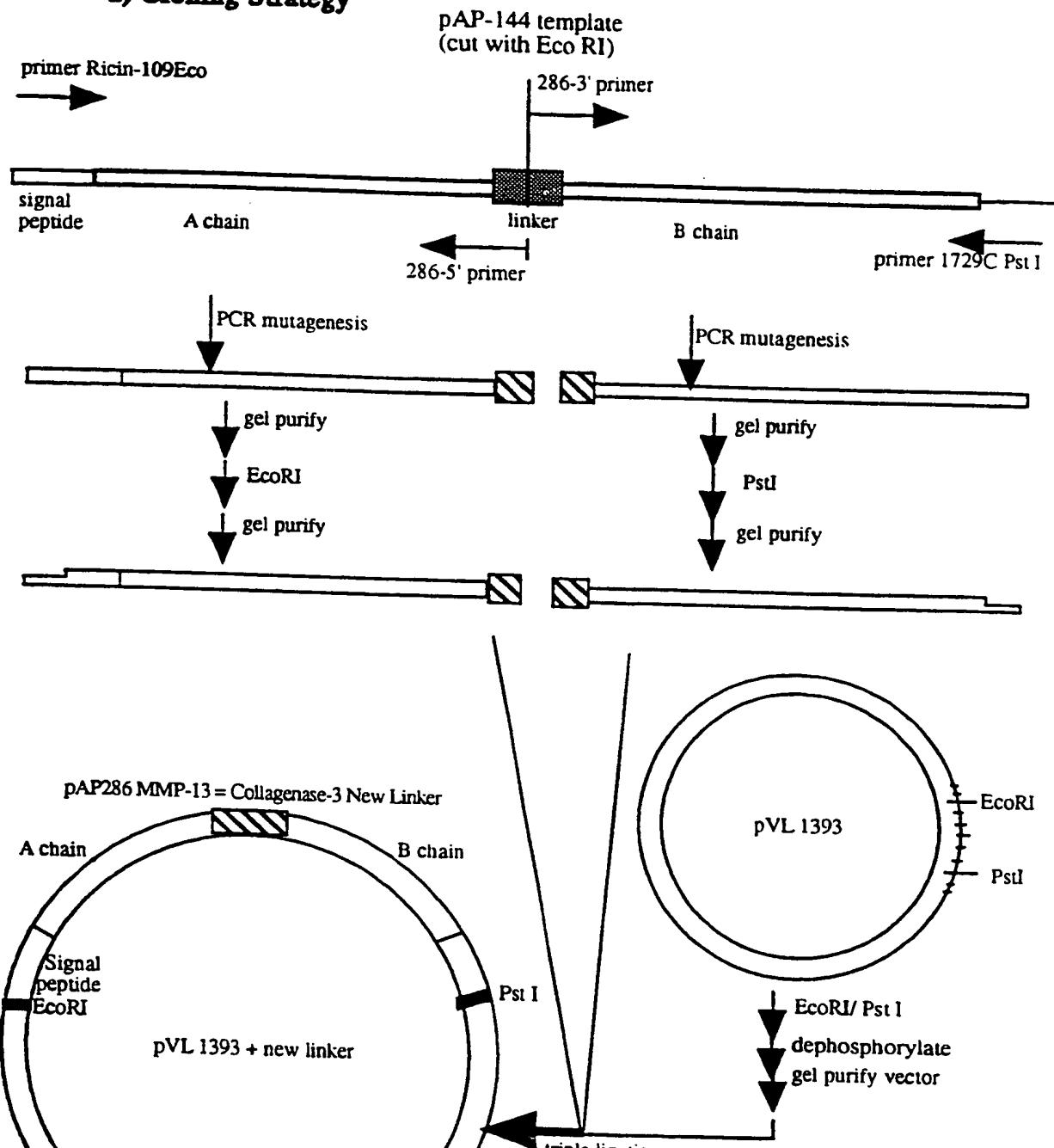
**Amino acid sequence Comparison of Mutant Preproricin Linker
region of MMP-11 (Stromelysin-3) to Wild Type**

Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-284 (MMP-11) linker:

A chain- H G P E G L R V G F Y E S D V M G R G H A R L V H V E E P H T -B chain

199/254

FIGURE 42A**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy****SUBSTITUTE SHEET (RULE 26)**

200/254

FIGURE 42B**Sequence of MMP-13 = Collagenase-3 Linker Region****WT preprocin linker**

primer 286-3'
 5' - GGTCAACGAGGCATTGTCGCTGATGTTGT - 3'
 * * * * * * * * * * * * * * * * * * * * * * * * * *
 -----TCTTGCTTATAAGGCCA|GTGGTACCAAATTTAAT-----
 -----AGAAACGAATATTCCGGT|CACCATGGTTAAAATTA-----
 * * * * * * * * * * * * * * * * * * * * * * * * * *
 3' - AGCAGTGTCAAACCTGGAGTCCCCAACGA - 5'
 primer 286-5'

1) PCR mutagenesis

2) Ligate with pVL1393

**pAP 286 linker
(MMP-13 variant)**
 -----GGACCTCAGGGGCTTGCT|GGTCAACGAGGCATTGTC-----
 -----CCTGGAGTCCCCAACGA|CCAGTTGCTCCGTAACAG-----

201/254

FIGURE 42C (P1)

Sequence of pAP286 insert

10 20 30 40 50

1 GAATTCATGAAACCAGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
 CTTAAGTACTTTGCCCTCCTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTGTTGGATCCACCTCAGGGTGGCTTACATTAG
 CCGTTGTACCGAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAAACTTACCA
 TCCTATTGTTGTATAAGGGTTGTTATGGGTTAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTGTCGCG
 CGCCCACGGTGACACGTTGATGTGTTGAAATAGTCTCGACAAGCGCC

201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATAACCAGTGGTGC
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGA
 ACTCTCAACCAAACGGATATTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGT
 ACCAATGCATA TTAGTACGTCGAAAGACAATGTAATCGCGAC
 CCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATT
 CCTTCATCCTGACA
 ACACCAGCCGATGGCACGACCTTATCGGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCAT
 CTTCACTGATGTTCAAAAT
 TAGTCCTCTACGTCTCGTTAGTGAGTAGAAAGTGA
 CTACAAGTTTA

451 CGATATACATTGCGCTTGGTGGTAATTATGATAGACT
 TGAAACAAC
 TTGC
 GCTATATGTAAGCGGAAACCACCA
 ATTAAACTATCTGA
 ACTTTGAACT

501 TGGTAATCTGAGAGAAAATATCGAGTG
 GGGAAATGGTCCACTAGAGGAGG
 ACCATTAGACTCT
 TTTATAGCTCA
 ACCCTTAC
 CAGGTGATCT
 CCTCC

551 CTATCTCAGCGCTTTATTACAGTACTGGTGGCACT
 CAGCTCCA
 ACT
 GATAGAGTC
 CGGAAATAATA
 ATGTC
 ATGAC
 CACC
 CGTGAGTC
 GAAGGTTGA

601 CTGGCTCGTCTTATAATTGCAT
 CCAATG
 ATT
 CAG
 CAGCAAG
 GAC
 CGAG
 CAAG
 GAAAT
 ATTAA
 CGT
 TAGGTT
 ACT
 AAAGT
 CTT
 CGTC
 GTTC

651 ATTCCAATATATTGAGGGAGAAAT
 GCGCAC
 GAGAATT
 TAGGT
 ACAAC
 CGGA
 TAAGGTT
 ATA
 ACT
 CCCT
 TTT
 AC
 GCGT
 GCT
 CTT
 AA
 AT
 CC
 AT
 GTT
 GGGCCT

701 GATCTGCACCAGATCCTAGCGTA
 ATT
 AC
 ACT
 TTG
 GAGA
 ATAG
 TTGGGG
 GAGA

202/254

FIGURE 42C (P2)

CTAGACGTGGTCTAGGATCGCATTAAATGTGAACCTTATCAACCCCTCT
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT
 GAAAGGTGACGTTAAGTTCTCAGATTGGTCCTCGAAACGATCAGGTTA
 801 TCAACTGCAAAGACGTAATGGTCAAATTCAAGTGTACGATGTGAGTA
 AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCATGGTGTATAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT
 901 TCGTCACAGTTGGACCTCAGGGCTTGCTGGTCAACGAGGCATTGTCGC
 AGCAGTGTCAAACCTGGAGTCCCCAACGACCAGTTGCTCCGTAACAGCG
 951 TGATGTTGTATGGATCCTGAGCCCATACTGCGTATCGTAGGTGAAATG
 ACTACAAACATACTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACTACCTACCTTCTAAGGTGTTGCCCTTGCCTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTCACTAGATTATGTCAGTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAAATGGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
 1151 GGTACAGTCGGGAGTCTATGTGATGATCTATGATTGCAAAACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATAACTAACGTTATGACGACGT
 1201 ACTGATGCCACCGCTGGCAAATATGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCTTACCTTGGTAGTATTTAGG
 1251 CAGATCTAGTCTAGTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAATCGTCGCTGAGTCCCTGTCACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTATGCCGTTAGTCAAGGTGGCTTCTACT
 AATGTCACGTTGGTTGAAATACGGCAATCAGTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTCGTTATCACCTGTTACACCTATCTCCTGACATCGTCACTTT
 1451 AGGCTGAACAAACAGTGGCTCTTATGCAGATGGTCAATACGTCCTCAG
 TCCGACTTGGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCCTACAAGTGATTCTAATACGGAAACAGT

203/254

FIGURE 42C (P3)

GTTTGCGCTATTACCGGAATGTTCACTAAGATTATGCCCTTGTCA

1551 TGTTAACGATCCTCTCTGTGGCCCTGCATCCTCTGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACC GGTTGCTACCTACA1601 TCAAGAACATGATGGAACCATTTAAATTGTATAGTGGATTGGTAGAT
AGTTCTTACTACCTTGGTAAAATTAAACATATCACCTAACCAACAACTCA1651 GTGAGGCGATCGGATCCGAGCCTAAACAAATCATTCTTACCCCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTGATAGACAGATTACT
ACCACTGGGTTTGGTTATACCAATGGTAATAAAACTATCTGTCTAATGA1751 CTCTTGCAGTGTGTGTGCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTT1801 GGACATTGTAATTTGTAAGTCAAAGGACAGCAAGTTATCGAATTCC
CCTGTAACATTTAAACATTGACTTCCTGTCGTTCAATATAGCTTAAGG1851 TGCAG
ACGTC

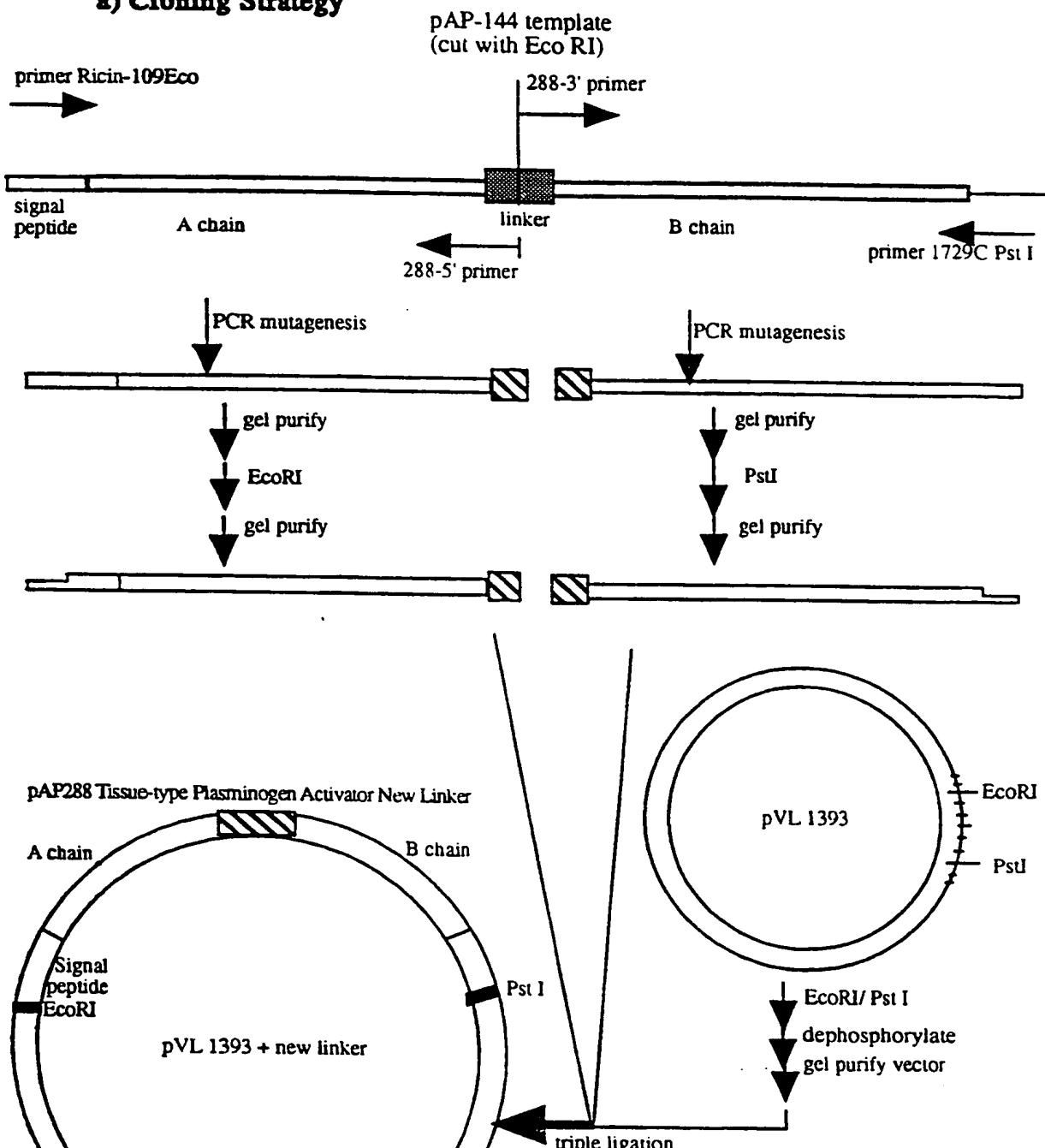
204/254

FIGURE 42D

**Amino acid sequence Comparison of Mutant Preproricin Linker
region of MMP-13 (Collagenase-3) to Wild Type**

Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain
pAP-286 (MMP-13) linker: A chain- G P Q G L A G Q R G I V -B chain

205/254

FIGURE 43A**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

206/254

FIGURE 43B**Sequence of Tissue-type Plasminogen Activator (tPA) Linker Region****WT preprocin linker**

primer 288-3'

5' - GGT CGT AAAG CT CTT GAAG CT GAT GTT GT - 3'
***** * * *

----- TCT TTG CTT ATA AGG CCA | GTGGT ACCAA ATT TAAT -----

----- AGAAA AC GAAT ATT CCG GT | CAC CAT GGTT AAA ATTA -----

3' - AGC AGT GT CAA ACC GC CT AG ACC CG TT CC - 5'
primer 288-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 288 linker**(tPA variant)**

----- GGCGG AT CT GGG CAA AGG | GGTCGT AAAG CT CTT GAA -----

----- CCG CCT AG ACC CG TT CC | CCAGC ATT CGAGA ACTT -----

207 / 254

FIGURE 43C (P1)

Sequence of pAP288 insert

	10	20	30	40	50
1	GAATT CATGAA ACCGGGAGGAA ATACT ATTGT AATATGGAT GTATGCAGT				
	CTTAAG TACT TTGCCCT CTTATGATAAC ATTACCTACATACGTCA				
51	GGCACACATGGCTTGTGATCCACCTCAGGGTGGTCTTCACATTAG				
	CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCA				
	TCCTATTGTTGTATAAGGGTTTGTTATGGTTAATATTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTGTTCGCGG				
	CGCCCACGGTGACACGTTGATGTGTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT				
251	ACAGAGTTGGTTGCCTATAAACCAACGGTTATTAGTTAGTTGAACTCTCA				
	TGTCTCAACCAACGGATATTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCAACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGTGGAAATAGCGCATATTTCTTCATCCTGACA				
	ACACCAGCCGATGGCACGACCTTATCGCGTATAAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATTTCACTGATGTTCAAAAT				
	TAGTCCTTCTACGTCTCGTTAGTAGAGAAAGTGAUTACAAGTTTA				
451	CGATATACATTGCCCTTGGTGGTAATTATGATAGACTTGAACAACTTGC				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTATAGCTCAACCCTTACCAAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTATTATTACAGTACTGGTGGCACTCAGCTTCCA				
	ACTGATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCTTATAATTGCAATCCAAATGATTTCAGAACGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTCGTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTACCGTGTCTTAATCCATGTTGGCCT				
701	GATCTGCACCAGATCCTAGCGTAATTACACTTGAAGAATAGTTGGGGAGA				

208/254

FIGURE 43C (P2)

CTAGACGTGGCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT
 GAAAGGTGACGTTAAGTCTCAGATTGGTCCTCGGAAACGATCAGGTTA
 801 TCAACTGCAAAGACGTAATGGTCCAAATTCAAGTGTACGATGTGAGTA
 AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT
 901 TCGTCACAGTTGGCGGATCTGGGAAAGGGGTCGTAAAGCTCTGAAGC
 AGCAGTGTCAAACCGCCTAGACCCGTTCCCCAGCATTGAGAACCTCG
 951 TGATGTTGTATGGATCCTGAGCCCATAAGTGCATCGTAGGTGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC
 1001 GTCTATGTGTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACAAATCCCTACCTTCTAAGGTGTTGCCCTTGCATTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTACGATTATGTCTACGTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATAACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCGCTGGCAAATATGGGATAATGGAACCACATCAAATCC
 TGACTACGGTGGCGACCGTTATACCCATTACCTTGGTAGTATTAGG
 1251 CAGATCTAGTCTAGTTTAGCAGCGACATCAGGAAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAATCGCGCTGTAGTCCCTGTCACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTATGCCGTTAGTCAGGTTGGCTTCTACT
 AATGTCACGTTGGTTGAAATACGGCAATCAGTCCAAACCGAAGGATGA
 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTCGTTATCACCTGTCATACCTATCTCCTGACATCGTCACTTT
 1451 AGGCTGAACAAACAGTGGCTCTTATGCAGATGGTTCAATACTGCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCCTACAAGTGATTCTAATACGGAAACAGT

209/254

FIGURE 43C (P3)

GT~~T~~TTGGCTCTATTAAACGGAATGTTCACTAAGATTATGCCCTTGTCA
1551 TGT~~A~~AGATCCTCTCTGTGGCCCTGCATCCTCTGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
1601 TCAAGAATGATGGAACCATTTAAATTTGTATAGTGGATTGGTGTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCAACAACTCA
1651 GTGAGGCGATCGGATCCGAGCCTAAACAAATCATTCTTACCCCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT
1701 TGGTGACCAAACCAAATATGGTTACCATTATTTGATAGACAGATTACT
ACCACTGGGTTGGTTACCAATGGTAATAAAACTATCTGTCTAATGA
1751 CTCTTGCAGTGTGTGTGCCTGCCATGAAAATAGATGGCTAAATAAAAAA
GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTT
1801 GGACATTGTAAATTTGTAAGTGAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTAAAACATTGACTTCTGTCGTTCAATATAGCTTAAGG
1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP288

210/254

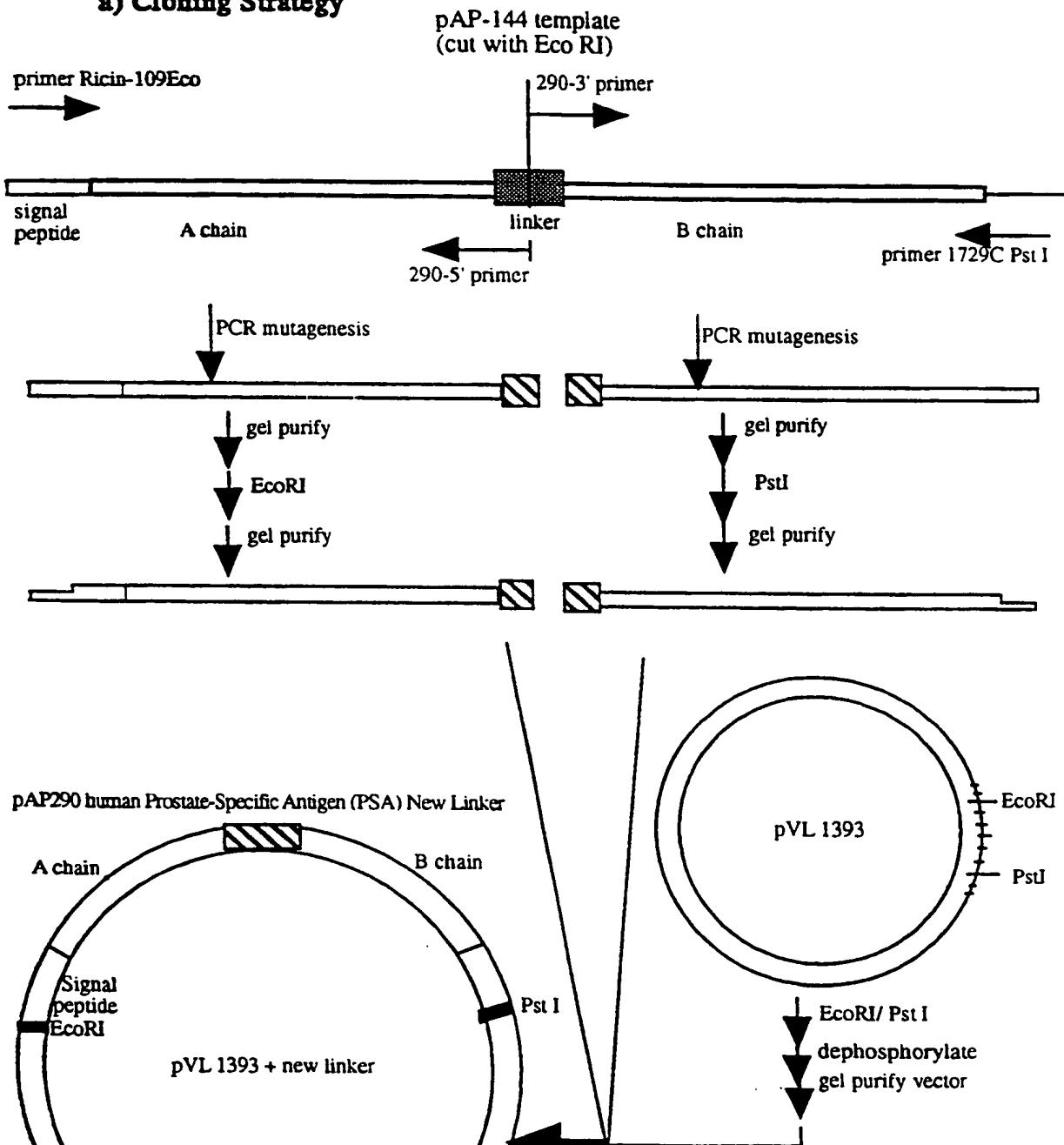
FIGURE 43D

**Amino acid sequence Comparison of Mutant Preprorocin Linker
region of Tissue-type Plasminogen Activator (tPA) to Wild Type**

Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-288 (tPA) linker: A chain- G G S G Q R G R K A L E-B chain

211/254

FIGURE 44A**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy****SUBSTITUTE SHEET (RULE 26)**

212/254

FIGURE 44B**Sequence of human Prostate-Specific Antigen (PSA) Linker Region****WT preprocin linker**

primer 290-3'
5' - TCTTCCGATATTTTAATGCTGATGTTGT - 3'
***** * *

-----TCTTGCTTATAAGGCCA|GTGGTACCAAATTTAAT-----
-----AGAAACGAATATTCCGGT|CACCATGGTTAAAATTA-----
***** * *

3' - AGCAGTGTCAAAAGAACAGTCGAGAAGAG - 5'
primer 290-5'

1) PCR mutagenesis

2) Ligate with pVL1393

**pAP 290 linker
(PSA variant)**

-----TCTTGTCAGCTCTCTC|TCTTCCGATATTTAAT-----
-----AGAAACAGTCGAGAAGAG|AGAAGGCTATAAAAATTA -----

213/254

FIGURE 44C (P1)

Sequence of pAP290 insert

10	20	30	40	50
1 GAATTCATGAAACCAGGGAGGAAATACATTGTAAATATGGATGTATGCAGT CTTAAGTACTTGGCCCTCCTTATGATAACATTATACTACATACGTCA				
51 GGCAACATGGCTTGTGATCCACCTCAGGGTGGCTTTCACATTAG CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC				
101 AGGATAACAACATATTCCCCAAACAATACCCATTATAAACTTACCA TCCTATTGTTGTATAAGGGTTGTTATGGTTAATATTTGAAATGGTGT				
151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTGTCGCC CGCCCACGGTGACACGTTGATGTGTTGAAATAGTCTCGACAAGCGCC				
201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATAACCAGTGTGCCAA AGCAAATTGTTGACCTCGACTACACTCTGACTATATGGTCACAACGGTT				
251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACCTCA TGTCTCAACCAAACGGATATTGGTTGCCAATAAAATCAACTTGAGAGT				
301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTCTTCATCCTGACA ACACCAGCCGATGGCACGACCTTATCGGTATAAAGAAAGTAGGACTGT				
401 ATCAGGAAGATGCAGAAGCAACTCATCTTCACTGATGTTCAAAAT TAGTCCTTCTACGTCTCGTTAGTGAGTAGAAAAGTGAACAGTTTTA				
451 CGATATACATTGCCTTGGGTTAATTATGATAGACTTGAACAACTTGC GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG				
501 TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG ACCATTAGACTCTTTATAGCTCAACCCTTACCAAGGTGATCTCCTCC				
551 CTATCTCAGCGCTTATTACAGTACTGGTGGCACTCAGCTCCA GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601 CTGGCTCGTCCCTTATAATTGCATCCAAATGATTTCAGAACAGCAAG GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTGTTC				
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA TAAGGTTATATAACTCCCTTTACCGTGCTCTTAATCCATGTTGGCCT				
701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA				

214/254

FIGURE 44C (P2)

CTAGACGTGGCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT
 GAAAGGTGACGTTAAGTTCTCAGATTGGTCCTCGAAACGATCAGGTTA
 801 TCAACTGCAAAGACGTAATGGTCCAATTCAAGGTTAAGTCACACATGCTACACTCAT
 AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCTCATGGGTATAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT
 901 TCGTCACAGTTCTTCAGCTCTCTCTCCGATATTTAATGC
 AGCAGTGTAAAAGAACAGTCGAGAAGAGAAGGCTATAAAATTACG
 951 TGATGTTGTATGGATCCTGAGGCCATAGTCGCTATCGTAGGTGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC
 1001 GTCTATGTGTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACCAATCCCTACCTTAAGGTGTTGCCCTTGCATTAT
 1051 CAGTTGTGGCCATGCAAGTCTAACATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTCAAGATTGTTACGTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAACATGGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
 1151 GGTACAGTCGGGAGTCTATGTGATGATCTATGATTGCAAACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCGCTGGCAAATATGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCCATTACCTGGTAGTATTAGG
 1251 CAGATCTAGTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTGTCACCATGGTGTG
 1301 TTACAGTCAAACCAACATTATGCCGTTAGTCAGGTTGGCTTCCTACT
 AATGTCACGTTGGTTGAAACGGCAATCAGTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTGTTACAACCATTGTTGGCTATATGGCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTAAA
 GAACGTTCGTTATCACCTGTCATACCTATCCCTGACATCGTCACTT
 1451 AGGCTGAACAACAGTGGCTTTATGCAGATGGTCAATACGTCCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGAAACAGT

215/254

FIGURE 44C (P3)

GTGTTGGCTCTATTAACGGAATGTTCACTAAGATTATGCCCTTGTCA
1551 TGTAAAGATCCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCAGGTTGCTACCTACA
1601 TCAAGAATGATGGAACCATTAAATTGTATAGTGGATTGGTAGAT
AGTTCTTACTACCTGGTAAAATTAAACATATCACCTAACCAACATCTA
1651 GTGAGGCGATCGGATCCGAGCCTAAACAAATCATTCTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT
1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTGATAGACAGATTACT
ACCACTGGGTTGGTTATACCAATGGTAATAAAACTATCTGTCTAATGA
1751 CTCTTGCACTGTGTGTGCCTGCCATGAAAATAGATGGCTAAATAAAAAA
GAGAACGTACACACACAGGACGGTACTTTATCTACCGAATTATTTT
1801 GGACATTGTAATTTGTAAGTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTAAACATTGACTTCCTGTCGTTCAATATAGCTTAAGG
1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP290

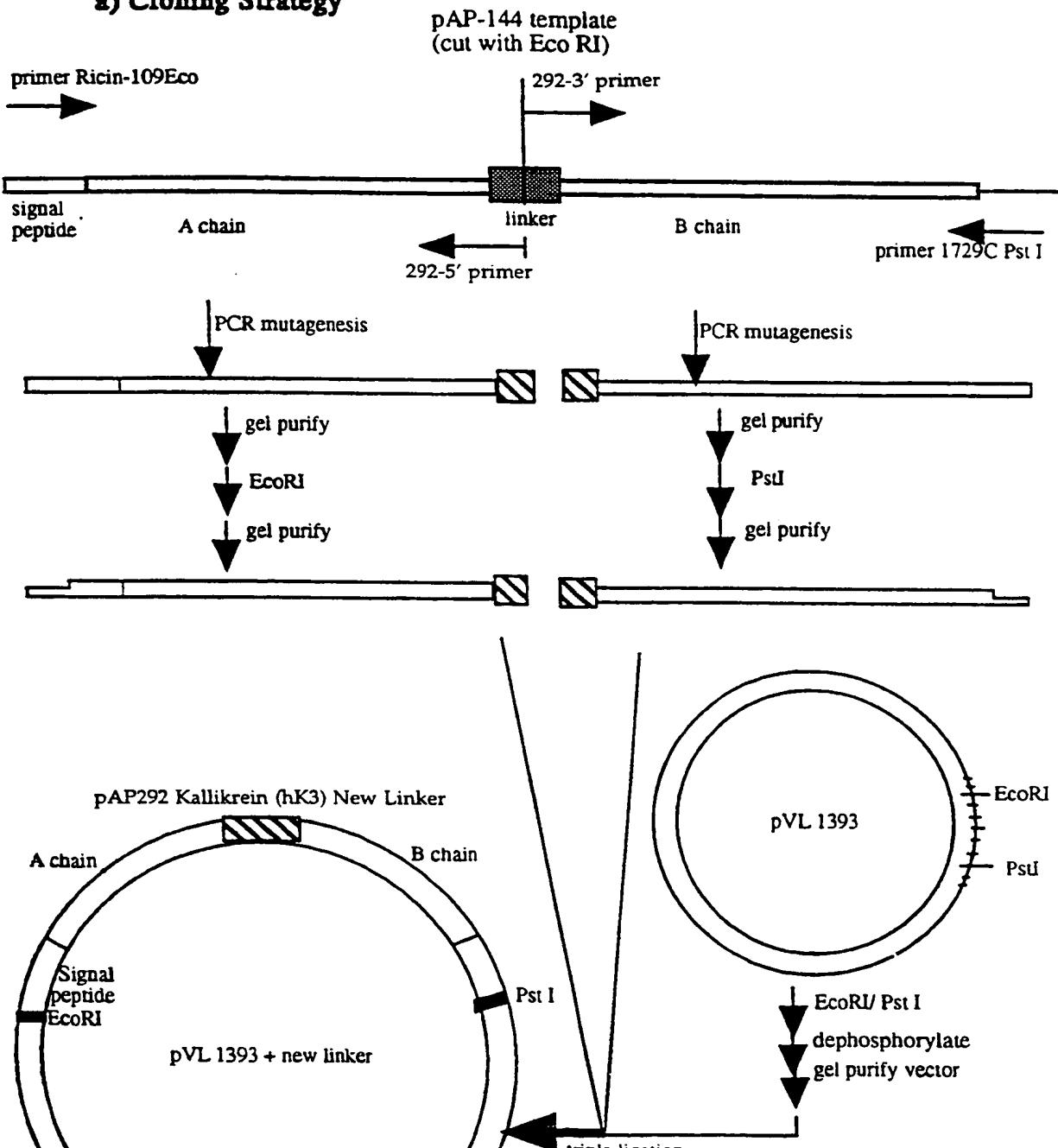
216/254

FIGURE 44D

**Amino acid sequence Comparison of Mutant Preproricin Linker
region of human Prostate-Specific Antigen (PSA) to Wild Type**

Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain
pAP-290 (PSA) linker: A chain- S L S A L L S S D I F N -B chain

217/254

FIGURE 45A**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

218/254

FIGURE 45B**Sequence of Kallikrein (hK3) Linker Region****WT preprocin linker**

primer 292-3'

5' - ATTATCGTGGCTTTAATGCTGATGTTGT - 3'

* * * *****

-----TCTTTGCCTTATAAGGCCA| GTGGTACCAAATTAAAT-----

-----AGAAACGAATATTCCGGT | CACCATGGTTAATTA-----

* * * ***

3' - AGCAGTGTCAAAAGAAACGGATCTAAATT - 5'

primer 292-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 292 linker

(Kallikrein variant)

-----TCTTTGCCTAGATTAAA| ATTATCGTGGCTTTAAT-----

-----AGAAACGGATCTAAATT | TAATAGCCACCGAAATTA-----

219/254

FIGURE 45C (P1)**Sequence of pAP292 insert**

10	20	30	40	50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTGGCCCTCCTTATGATAACATTACCTACATACGTCA

51 GGCAACATGGCTTGTTGGATCCACCTCAGGGTGGCTTACATTAG
CCGTTGACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC

101 AGGATAACAACATATTCCCCAAACAATACCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGTTGTTATGGGTTAATATTGAAATGGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTGCCGG
CGCCCACGGTGACACGTTCGATGTGTTGAAATAGTCTCGACAAGCGCC

201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATAACCAAGTGTGCAA
AGCAAATTGTTGACCTCGACTACACTCTGACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACCTCTCA
TGTCTCAACCAAACGGATATTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTCTTCATCCTGACA
ACACCAGCCGATGGCACGACCTTATCGGTATAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTCACTGATGTTCAAAAT
TAGTCCTTCTACGTCTCGTTAGTAGAAAAGTAGACTACAAGTTTA

451 CGATATACATTGCCCTTGGTGGTAATTATGATAGACTTGAACAACTTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTATAGCTAACCCCTTACCAAGGTGATCTCCCTCC

551 CTATCTCAGCGCTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTCTTATAATTGCATCCAAATGATTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTACTAAAGTCTCGTCGTTTC

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTATATAACTCCCTCTTACCGTGCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA

220/254

FIGURE 45C (P2)

CTAGACGTGGCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT

751 CTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTCCTCGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTCCAATTAGTGTGTACGATGTGAGTA
AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCATGGTGTATAAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCAACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTCTTGCCCTAGATTTAAAATTATCGGTGGCTTAATGC
AGCAGTGTCAAAAGAACGGATCTAAATTAAAGCCACCGAAATTACG

951 TGATGTTGTATGGATCCTGAGCCCAGTAGTGCATCGTAGGTCGAAATG
ACTACAAACATACTACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATAACACAACATACAATCCCTACCTTCTAAGGTGTTGCCCTTGCCTTAT

1051 CAGTTGTGGCCATGCAAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTAGATTATGTCACGTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC

1151 GGTACAGTCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTAGATACTAACGTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCACATCAAATCC
TGACTACGGTGGCGACCGTTATACCCATTACCTTGGTAGTATTAGG

1251 CAGATCTAGTCTAGTTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
GTCTAGATCAGATCAAATCGTCGTAGTCCCTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTATGCCGTTAGTCAGGTTGGCTTCTACT
AATGTCACGTTGGTTGTAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTGTTACAACCAATTGTTGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCGATATACCAAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTAAA
GAACGTTCGTTATCACCTGTCATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAAACAGTGGCTTTATGCAGATGGTCAATAACGTCCCTCAG
TCCGACTTGTGTCACCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGAAACAGT

221/254

FIGURE 45C (P3)

GTTTGGCTCTATTACCGGAATGTTACTAAGATTATGCCCTTGTCA
1551 TGTTAACGATCCCTCTTGTGGCCCTGCATCCTCTGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTGCTACCTACA
1601 TCAAGAACATGATGGAACCATTAAATTGTATAGTGGATTGGTAGAT
AGTTCTTACTACCTTGGTAAATTAACATATCACCTAACCAACATCTA
1651 GTGAGGCAGTCGGATCCGAGCCTTAAACAAATCATTCTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT
1701 TGGTGACCCAAACCAAATATGGTACCATTATTTGATAGACAGATTACT
ACCACTGGGTTTGGTTATACCAATGGTAATAAAACTATCTGTCTAATGA
1751 CTCTTGCAGTGTGTGTCCCTGCCATGAAAATAGATGGCTTAAATAAAAAA
GAGAACGTACACACACAGGACGGTACTTTATCTACCGAATTATTTT
1801 GGACATTGTAATTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTAAACATTGACTTCCGTGTTCAATATAGCTTAAGG
1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP292

222/254

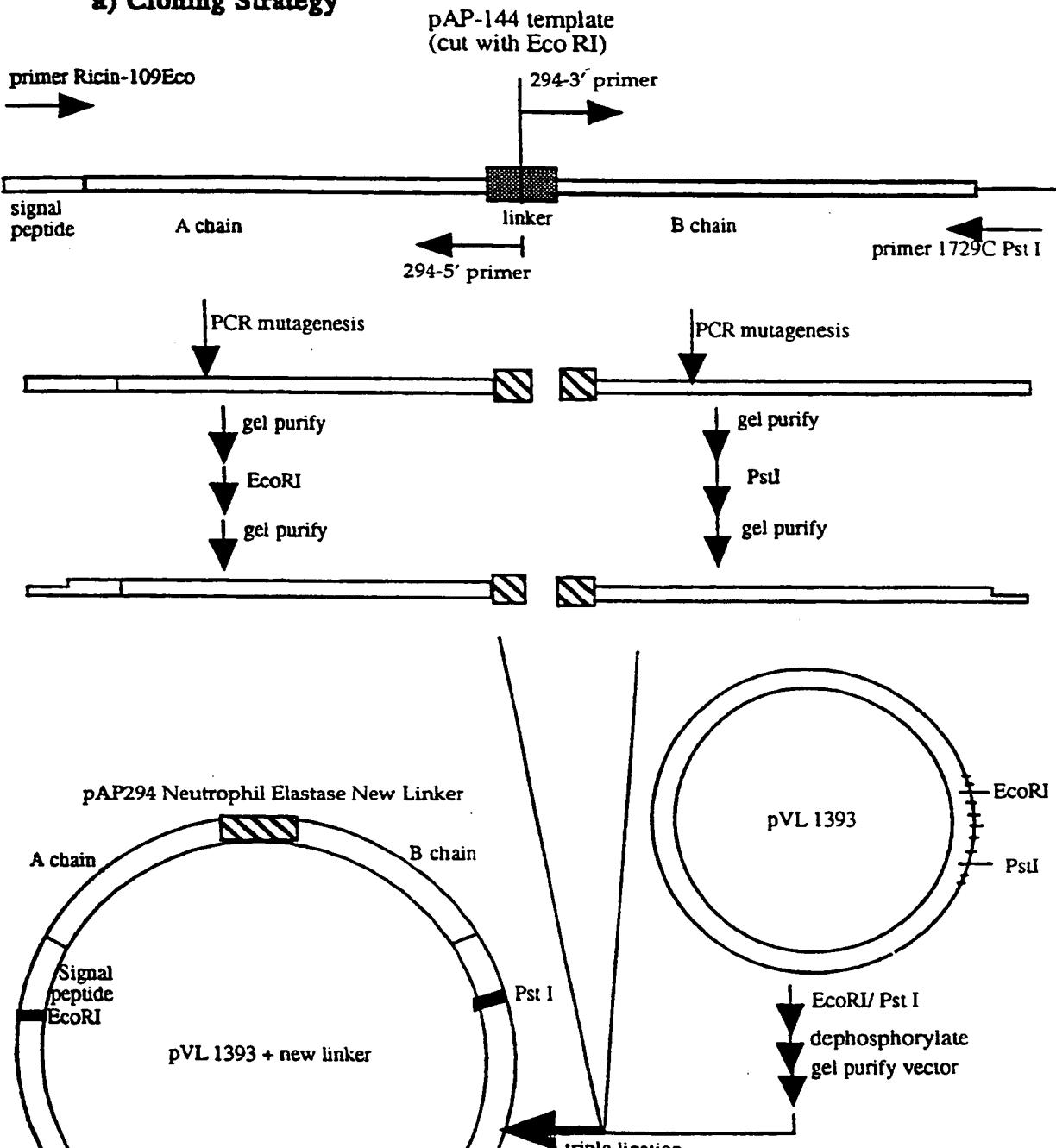
FIGURE 45D

Amino acid sequence Comparison of Mutant Preproricin Linker region of Kallikrein (hK3) to Wild Type

Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-292 (hK3) linker: A chain- S L P R F K I I G G F N -B chain

223/254

FIGURE 46A**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy****SUBSTITUTE SHEET (RULE 26)**

224/254

FIGURE 46B**Sequence of Neutrophil Elastase Linker Region****WT preprocin linker**

primer 294-3'

5' - GTTCCTGGTAATTTAATGCTGATGTTGT -3'
** *****

-----TCTTTGCTTATAAGGCCA|GTGGTACCAAATTTAAT-----

-----AGAAACGAATATTCCGGT|CACCATGGTTAAAATTA-----

*** *** *

3'-AGCAGTGTCAAAAGAAACGAACCGTAACGA -5'

primer 294-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 294 linker

(Neutrophil elastase variant)

-----TCTTTGCTTGGCATTGCT|GTTCCTGGTAATTTAAT-----

-----AGAAACGAACCGTAACGA|CAAGGACCATTAAAATTA-----

225/254

FIGURE 46C (P1)**Sequence of pAP294 insert**

10	20	30	40	50
1				
GAATTCATGAAACCGGGAGGAAATACTATTGTAAATATGGATGTATGCAGT				
CTTAAGTACTTTGCCCTCCTTATGATAACATTATACCTACATAACGTCA				
51				
GGCAACATGGCTTGGATCCACCTCAGGGTGGCTTACATTAG				
CCGGTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAA				
101				
AGGATAACAACATATCCCCAAACAATACCCAATTATAAACTTACCA				
TCCTATTGTTGTATAAGGGTTGTTATGGGTTAATATTGAAATGGTGT				
151				
GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTGTTCGCGG				
CGCCCACGGTGACACGTTGATGTGTTGAAATAGTCTCGACAAGCGCC				
201				
TCGTTAACAACTGGAGCTGATGTGAGACATGATATAACCAGTGTGCCAA				
AGCAAATTGTTGACCTCGACTACACTCTGACTATATGGTCACAACGGTT				
251				
ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACTCTCA				
TGTCTAACCAACGGATATTGGTGCCTAACAAACTTGAGAGT				
301				
AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTACCAATGCATA				
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351				
TGTGGTCGGTACCGTGCTGGAAATAGCGCATATTCTTCATCCTGACA				
ACACCAAGCCGATGGCACGACCTTATCGCGTATAAGAAAGTAGGACTGT				
401				
ATCAGGAAGATGCAGAAGCAATCACTCATCTTCACTGATGTTAAAAAT				
TAGTCCTCTACGTCTCGTTAGTGAGTAGAAAGTGAACACTACAAGTTTA				
451				
CGATATACATTGCCCTGGTGGTAATTATGATAGACTTGAACAACTTGC				
GCTATATGTAAGCGAAACCAACCATTAATACTATCTGAACCTGTTGAACG				
501				
TGGTAATCTGAGAGAAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG				
ACCATTAGACTCTTTATAGCTAACCCCTTACCAAGGTGATCTCCTCC				
551				
CTATCTCAGCGTTTATTACAGTACTGGTGGCACTCAGCTTCAA				
GATAGAGTCGCGAAATAATAATGTATGACCGTGAAGTCGAAGGTTGA				
601				
CTGGCTCGTTCTTATAATTGCATCAAATGATTCAGAAGCAGCAAG				
GACCGAGCAAGGAAATATTAACGTAGGTTACTAAAGTCTCGTCGTT				
651				
ATTCCAATATATTGAGGGAGAAATGCCACGAGAATTAGGTACAACCGGA				
TAAGGTTATATAACTCCCTCTTACCGTGCTTAATCCATGTTGGCCT				
701				
GATCTGCACCAAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA				

226/254

FIGURE 46C (P2)

CTAGACGTGGCTAGGATCGCATTAAATGTGAACCTTATCAACCCCTCT
 751 CTTTCACCGCAATTCAAGAGTCTAACCAAGGAGCTTGCTAGTCCAAT
 GAAAGGTGACGTTAAGTCTCAGATTGGTCCTCGAAACGATCAGGTTA
 801 TCAAACGTCAAAGACGTAATGGTCCAAATTCACTGTGTACGATGTGAGTA
 AGTTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCAATATCTACCGTGGAGGTGGT
 901 TCGTCACAGTTTCTTGCTTGGCATTGCTGTTCTGGTAATTAAATGC
 AGCAGTGTCAAAAGAAACGAACCGTAACGACAAGGACCATTAAAATTACG
 951 TGATGTTGTATGGATCCTGAGCCCATACTGCGTATCGTAGGTCGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAAACGAAACGCAATA
 CAGATACACAACATACAAATCCCTACCTTCTAAGGTGTTGCCCTTGCCTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTCAAGATTATGTCTACGTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
 CTTTCTCTGTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATAACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGCGACCGTTATACCTTACCTTGGTAGTATTAGG
 1251 CAGATCTAGTCTAGTTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTGTCACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTATGCCGTTAGTCAGGTTGGCTTCTACT
 AATGTCAGTTGGTTGTAATACGGCAATCAGTCCAAACCGAAGGATGA
 1351 AATAATACACAACCTTGTACAACCATTGTTGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTCGTTATCACCTGTTACACCTATCTCCTGACATCGTCACTT
 1451 AGGCTGAACAAACAGTGGCTTTATGCAGATGGTTCAATACGTCCCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCCTACAAGTGAATTCTAATATACGGGAAACAGT

227/254

FIGURE 46C (P3)

GTGTTGGCTCTATTAACGGAATGTTCACTAAGATTATGCCCTTGTCAT
1551 TGTTAACGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACAA
1601 TCAAGAACATGGAACCATTTAAATTGTATAGTGGATTGGTGTAGAT
AGTTCTTACTACCTTGGTAAAATTAAACATATCACCTAACCAACATCTA
1651 GTGAGGCGATCGGATCCGAGCCTAAACAAATCATCTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT
1701 TGGTGACCCAAACCAAATATGGTTACCATTTATTTGATAGACAGATTACT
ACCACTGGGTTGGTTATACCAATGGTAATAAAACTATCTGTCTAATGA
1751 CTCTTGCAGTGTGTGTGCCTGCCATGAAAATAGATGGCTAAATAAAAAA
GAGAACGTCACACACACAGGACGGTACTTTATCTACCGAATTATTTT
1801 GGACATTGTAATTTGTAACGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTAAAACATTGACTTCCCTGTCGTTCAATAAGCTTAAGG
1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP294

228/254

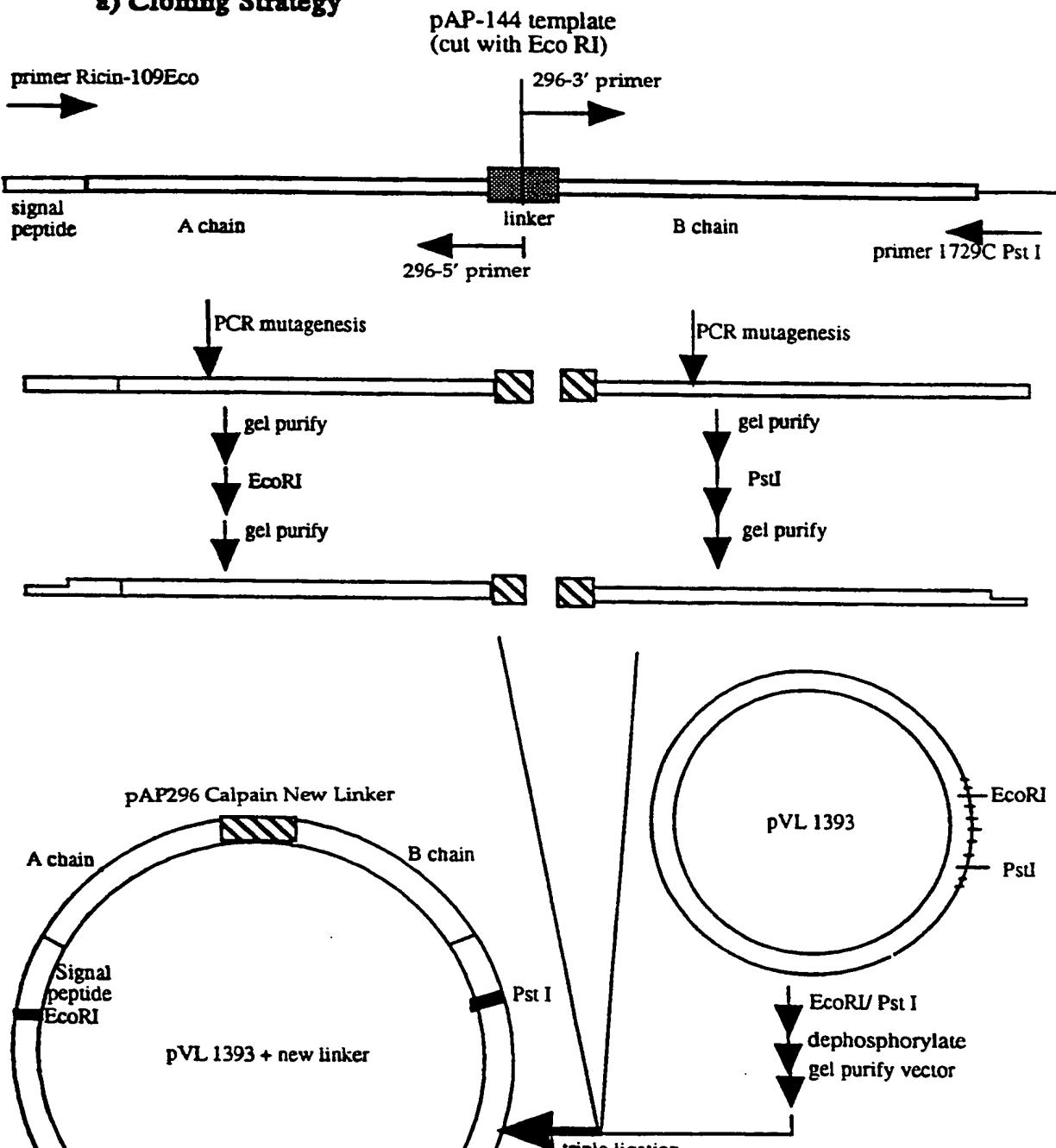
FIGURE 46D

Amino acid sequence Comparison of Mutant Preproricin Linker
region of Neutrophil elastase to Wild Type

Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-294 (Neutrophil elastase) linker:
A chain- S L L G I A V P G N F N -B chain

229/254

FIGURE 47A**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy****SUBSTITUTE SHEET (RULE 26)**

230/254

FIGURE 47B**Sequence of Calpain Linker Region****WT preprocin linker**

primer 296-3'

5' - ACTCCTAGAACCCCCCAGCTGATGTTGT - 3'

***** *****

----- TCTTTGCCTTATAAGGCCA | GTGGTACCAAATTAAAT -----

----- AGAACGAAATATTCCGGT | CACCATGGTTAAATTA -----

* * * * * 3' - AGCAGTGTCAAAAAAAAGTTTTTATAACAA - 5'

primer 296-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 296 linker

(Calpain variant)

----- TTTTCAAAAATATTGTT | ACTCCTAGAACCCCCCA -----

----- AAAAAGTTTTTATAACAA | TGAGGATCTGGGGGGGT -----

231/254

FIGURE 47C (P1)**Sequence of pAP296 insert**

10	20	30	40	50
1 GAATTCATGAAACCGGGAGGAATACTATTGTAATATGGATGTATGCAGT				
CTTAAGTACTTGGCCCTCCTTATGATAACATTACCTACATACGTCA				
51 GGCAACATGGCTTGTTGGATCCACCTCAGGGGGTCTTCACATTAG				
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAA				
101 AGGATAACAACATATTCCCCAACAACTACCAATTATAAACTTACCA				
TCCTATTGTTGTATAAGGGTTTGTATGGTTAATATTGAAATGGTGT				
151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTATCAGAGCTGTTCGCG				
CGCCCACGGTGACACGTTCGATGTGTTGAAATAGTCTCGACAAGCGCC				
201 TCGTTAACAACTGGAGCTGATGTGAGACATGATATACCAAGTGTGCCAA				
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT				
251 ACAGAGTTGGTTGCCTATAAACCAACGGTTATTTAGTTGAACTCTCA				
TGTCTAACCAAACGGATATTGGTTGCCAAATAATCAACTTGAGAGT				
301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
TTAGTACGTCTCGAAAGACAATGTAATCGCACCTACAGTGGTTACGTAT				
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTCATCCTGACA				
ACACCAGCCGATGGCACGACCTTATCGGTATAAAGAAAGTAGGACTGT				
401 ATCAGGAAGATGCAGAAGCAATCACTCATTTCACTGATGTTCAAAAT				
TAGTCCTTCTACGTCTCGTTAGTAGAAAAGTAGACTACAAGTTTA				
451 CGATATACATTGCCTTGGTGGTAATTATGATAGACTTGAACAACTTGC				
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG				
501 TGGTAATCTGAGAGAAAATATCGAGTTGGAAATGGTCCACTAGAGGAGG				
ACCATTAGACTCTTTATAGCTAACCTTACCAAGGTGATCTCCTCC				
551 CTATCTAGCGCTTTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601 CTGGCTCGTCTTATAATTGATCCAAATGATTCAGAAGCAGCAAG				
GACCGAGCAAGGAAATATTAACGTAGGTTACTAAAGTCTCGTCGTT				
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
TAAGGTTATATAACTCCCTTTACCGTGCTTTAATCCATGTTGGCCT				
701 GATCTGCACCAAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGAGA				

232/254

FIGURE 47C (P2)

CTAGACGTGGCTAGGATCGCATTAAATGTGAACCTCTTATCAACCCCTCT
 751 CTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTGCTAGTCCAAT
 GAAAGGTGACGTTAAGTCTCAGATTGGTCCTCGGAAACGATCAGGTTA
 801 TCAACTGCAAAGACGTAATGGTCAAATTCAAGTGTGTACGATGTGAGTA
 AGTGACGTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCCACATATCTACCGGTGGAGGTGGT
 901 TCGTCACAGTTTTTCAAAAATATTGTTACTCCTAGAACCCCCCAGC
 AGCAGTGTCAAAAAAAAGTTTATAACAATGAGGATCTGGGGGGTCG
 951 TGATGTTGTATGGATCCTGAGCCCAGTGCCTAGTGTAGGTGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATACAATCCCTACCTTCTAAGGTGTTGCCCTTGCCTTAT
 1051 CAGTTGTGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTAGATTATGTCACGTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAAACTGCTGCA
 CCATGTCAGGCCCTCAGATAACACTAGATAACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCACATCATAAATCC
 TGACTACGGTGGCGACCCTTATACCTTATTACCTTGGTAGTATTAGG
 1251 CAGATCTAGTCTAGTTTAGCAGCGACATCAGGGAACAGTGGTACCAACAC
 GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTGTCAACCAGGTGTG
 1301 TTACAGTGCACAAACACATTATGCCGTTAGTCAGGTTGGCTTCTACT
 AATGTCACGTTGGTTGTAACACGGCAATCAGTCCAAACCGAAGGATGA
 1351 AATAATACACAACCTTTGTTACAACCAATTGTTGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTAAA
 GAACGTTCGTTATCACCTGTTACACCTATCTCCTGACATCGTCACCTT
 1451 AGGCTGAACAAACAGTGGCTTTATGCAGATGGTCAAAACGTCCTCAG
 TCCGACTTGTGTCACCGAGAAACGTCACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCCTACAAGTGATTCTAATATACGGAAACAGT

233/254

FIGURE 47C (P3)

GTTCGGCTCTATTAACGGAATGTTCACTAAGATTATGCCCTTGTCA
1551 TGTAAAGATCCTCTCTGTGGCCCTGCATCCTCTGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
1601 TCAAGAACATGATGGAACCATTAAATTGTATAGTGGATTGGTAGAT
AGTTCTTACTACCTTGGTAAATTAAACATATCACCTAACCAACATCTA
1651 GTGAGGCAGTCGGATCCGAGCCTAAACAAATCATTCTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTGTTAGTAAGAAATGGGAGAGGT
1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTGATAGACAGATTACT
ACCACTGGGTTGGTTATACCAATGGTAATAAAACTATCTGTCTAATGA
1751 CTCTTGCAGTGTGTGTCTGCCATGAAAATAGATGGCTAAATAAAAAA
GAGAACGTACACACACAGGACGGTACTTTATCTACCGAATTATTTT
1801 GGACATTGTAATTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTAAAACATTGACTTCTGTCGTTCAATATAGCTTAAGG
1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP296

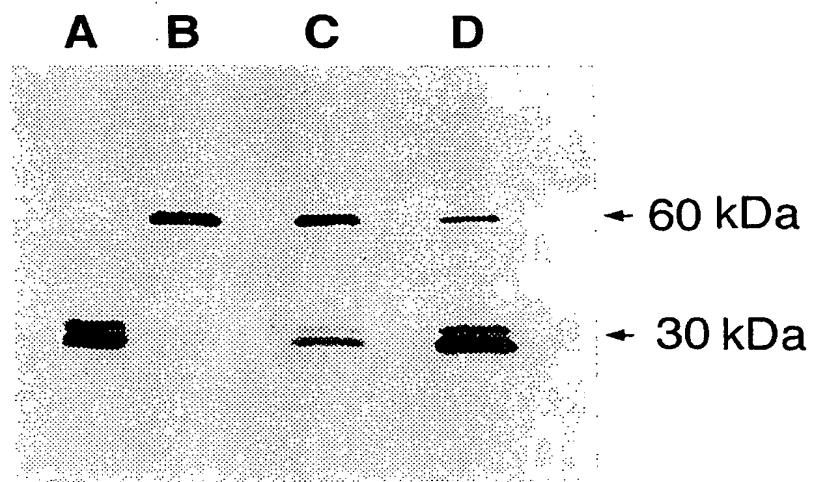
234/254

FIGURE 47D

**Amino acid sequence Comparison of Mutant Preproricin Linker
region of Calpain to Wild Type**

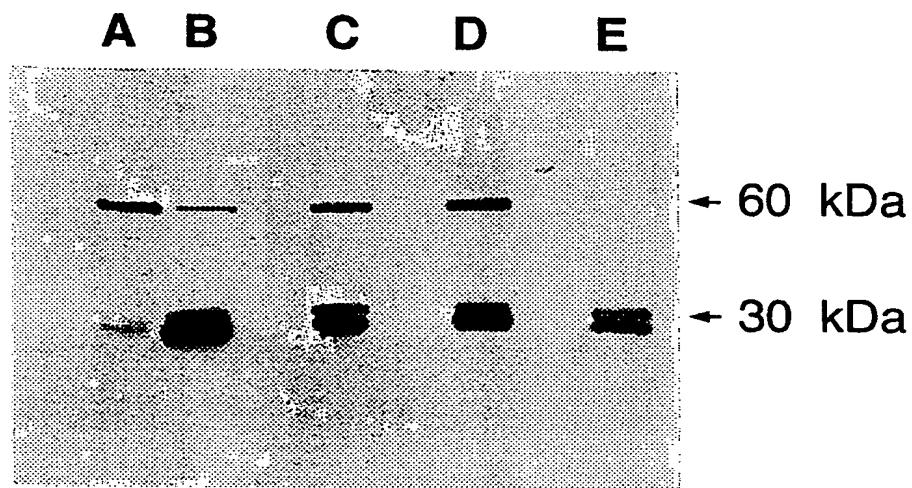
Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain
pAP-296 (Calpain) linker: A chain- F F K N I V T P R T P P -B chain

235/254

FIGURE 48**Cleavage of pAP 214 by Cathepsin B**

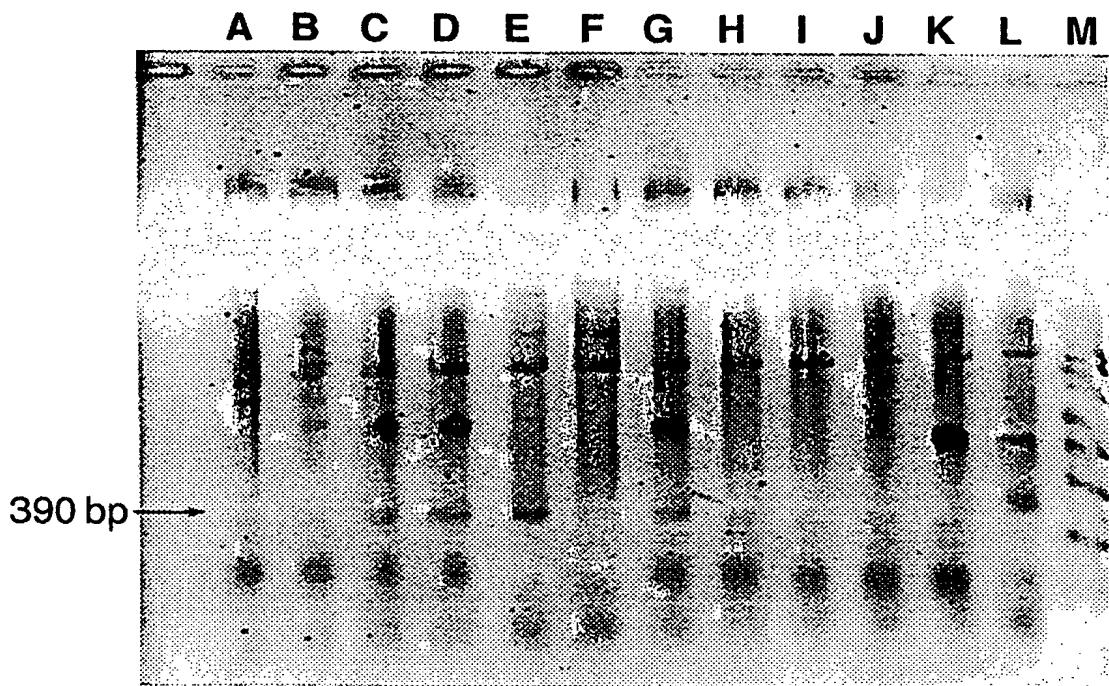
- A. Ricin standard**
- B. pAP 214**
- C. pAP 214 digested with 100 ng of Cathepsin B (18 hours)**
- D. pAP 214 digested with 618 ng of Cathepsin B (18 hours)**

236/254

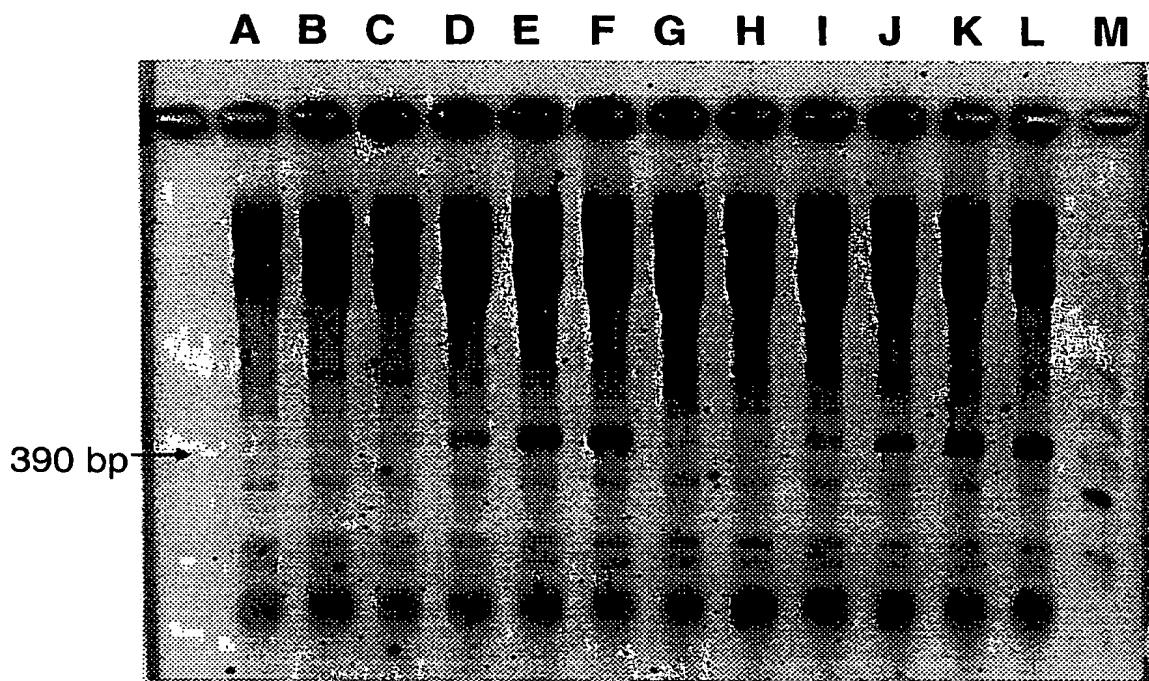
FIGURE 49**Cleavage of pAP 220 with MMP-9**

- A. pAP 220
- B. pAP 220 digested with 200 ng of MMP-9 (16 hrs)
- C. pAP 220 digested with 20 ng of MMP-9 (16hrs)
- D. pAP 220 digested with 20 ng of MMP-9 (2hrs)

237/254

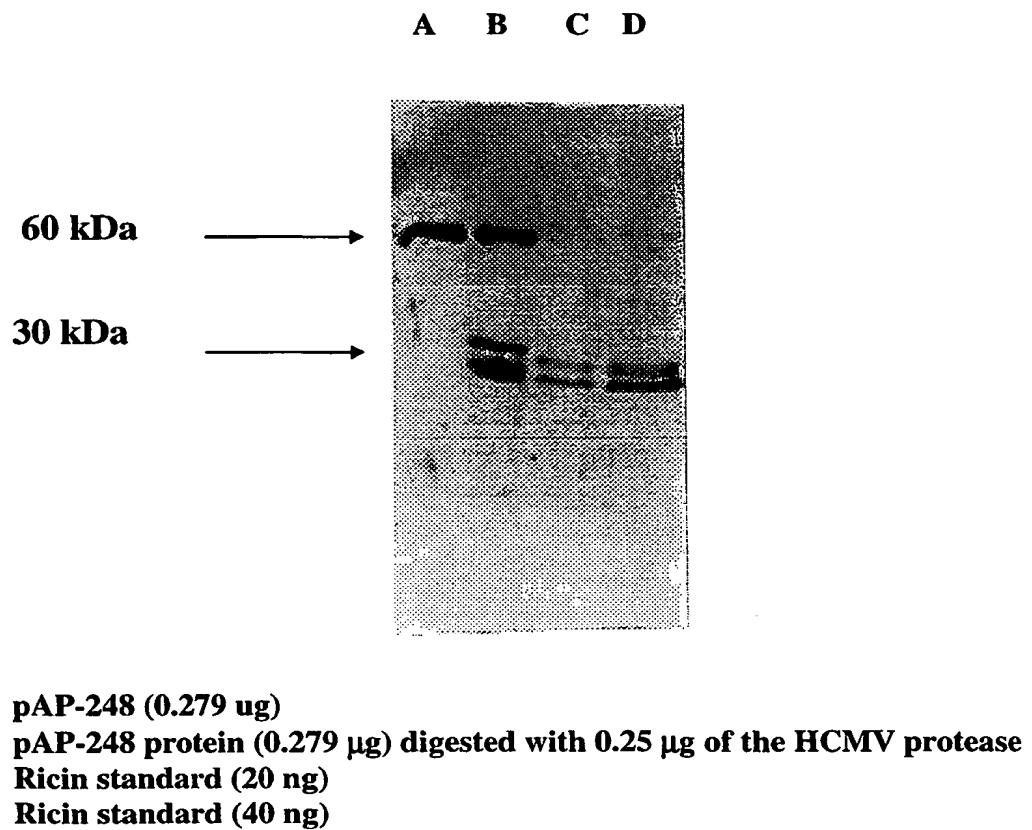
FIGURE 50**Activation of pAP 214**

- A. 41.7 pg of pAP 214 digested with Cathepsin B
- B. 291 pg of pAP 214 digested with Cathepsin B
- C. 2.0 ng of pAP 214 digested with Cathepsin B
- D. 14.2 ng of pAP 214 digested with Cathepsin B
- E. 100 ng of pAP 214 digested with Cathepsin B
- F. Negative control
- G. Ricin A chain
- H. 41.7 pg of pAP 214 variant
- I. 291 pg of pAP 214 variant
- J. 2.0 ng of pAP 214 variant
- K. 14.2 ng of pAP 214 variant
- L. 100ng of pAP 214 variant
- M. RNA ladder

FIGURE 51**Activation of pAP 220**

- A. 48.5 pg of pAP 220 variant
- B. 291 pg of pAP 220 variant
- C. 2.0 ng of pAP 220 variant
- D. 14.3 ng of pAP 220 variant
- E. 100 ng of pAP 220 variant
- F. Ricin A chain
- G. Negative Control
- H. 48.5 pg of pAP 220 variant digested with MMP-9
- I. 291 pg of pAP 220 variant digested with MMP-9
- J. 2.0 ng of pAP 220 variant digested with MMP-9
- K. 14.3 ng of pAP 220 variant digested with MMP-9
- L. 100 ng of pAP 220 variant digested with MMP-9
- M. RNA ladder

239/254

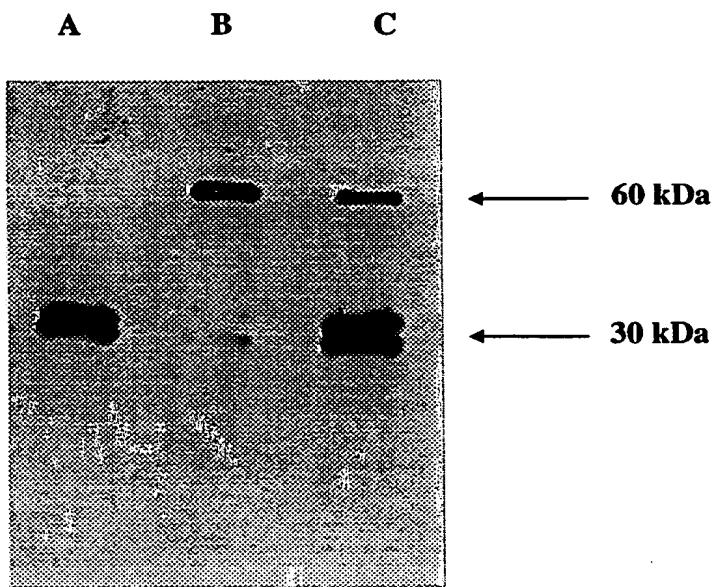
FIGURE 52**Cleavage of pAP-248 Protein by The Human Cytomegalovirus (HCMV) protease**

240/254

FIGURE 53**Activation of pAP-248 Protein**

- A. 90 ng of pAP-248 variant
- B. 12.8 ng of pAP-248 variant
- C. 1.8 ng of pAP-248 variant
- D. 260 pg pAP-248 variant
- E. 37 pg of pAP-248 variant
- F. Negative control
- G. Ricin A chain
- H. 37 pg of pAP-248 digested with HCMV protease
- I. 260 pg of pAP-248 digested with HCMV protease
- J. 1.8 ng of pAP-248 digested with HCMV protease
- K. 12.8 ng of pAP-248 digested with HCMV protease
- L. 90 ng of pAP-248 digested with HCMV protease
- M. RNA ladder

241/254

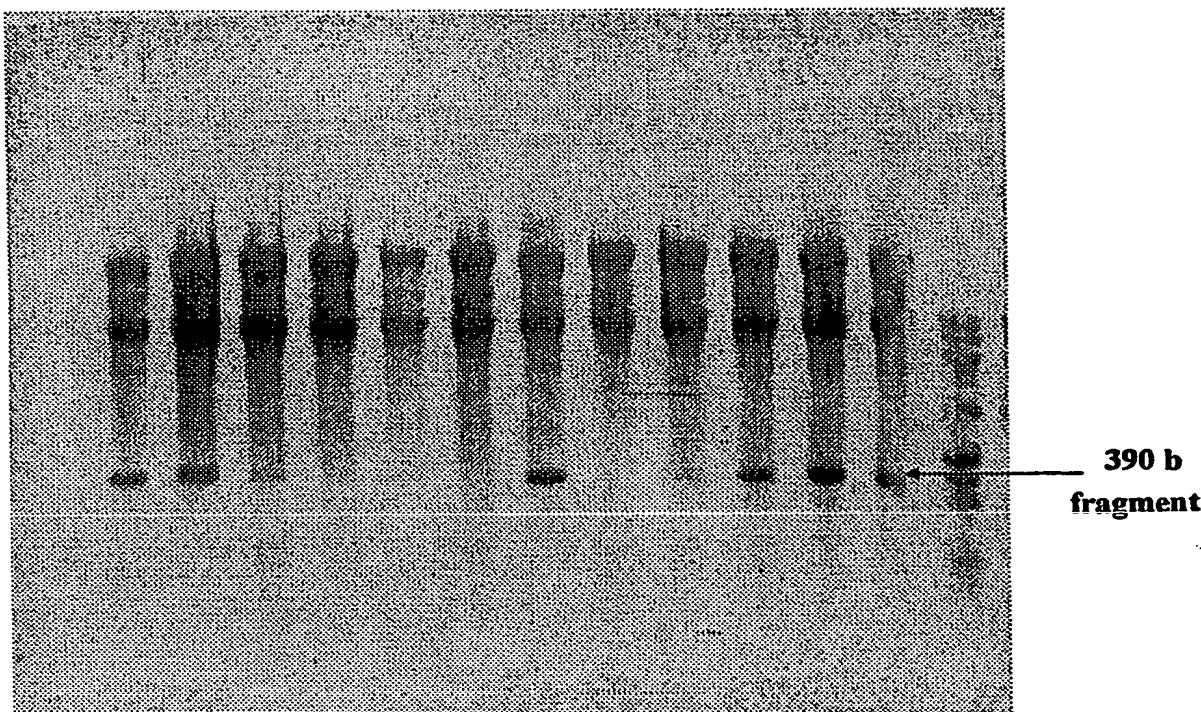
FIGURE 54**Cleavage of pAP-256 protein by The Hepatitis A Virus 3C (HAV 3C) Protease**

- A. Ricin standard (0.250 ug)
- B. pAP-256 protein (0.378 ug)
- C. pAP-256 protein digested (0.302 ug) with 1.25 µg of the HAV 3C protease

242/254

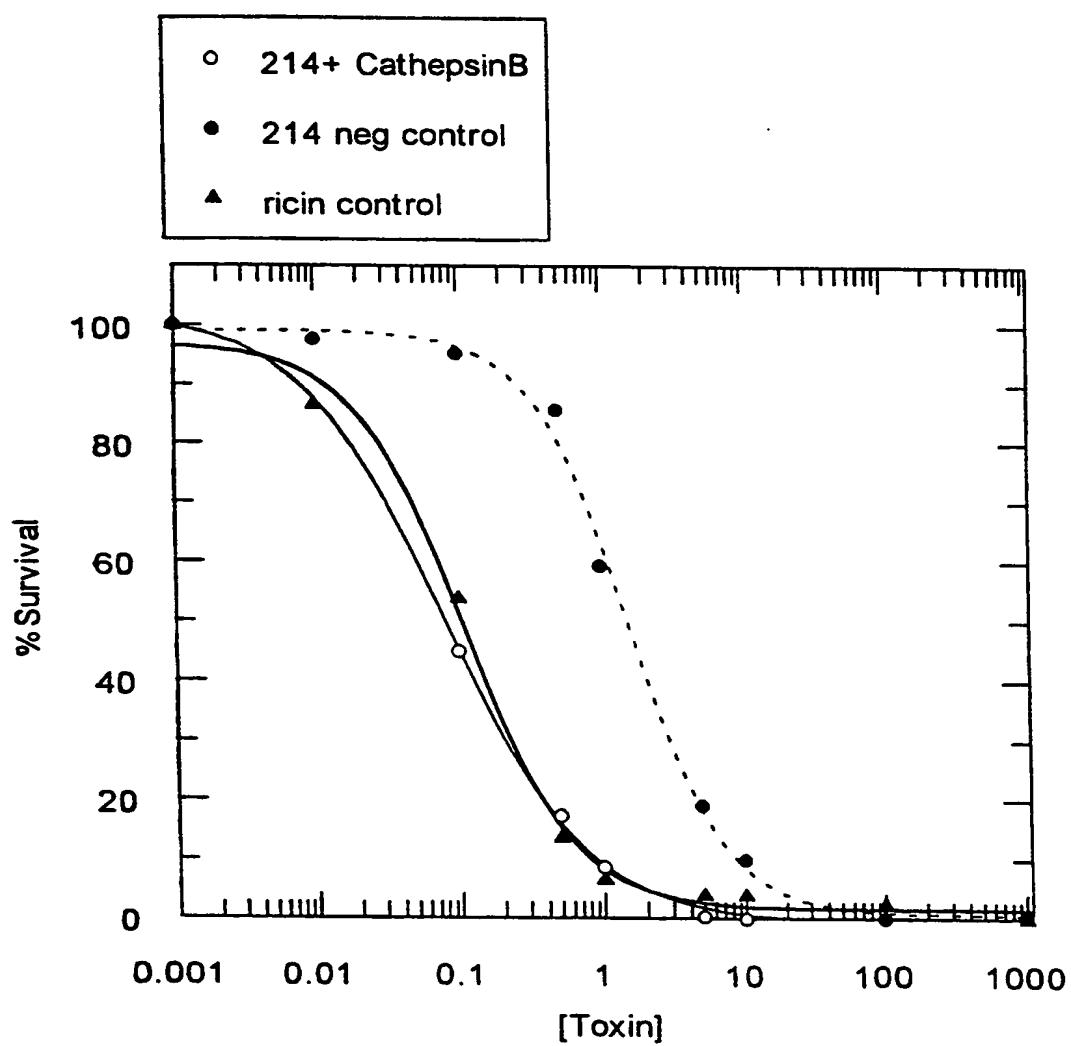
FIGURE 55**Activation of pAP-256 Protein**

A B C D E F G H I J K L M



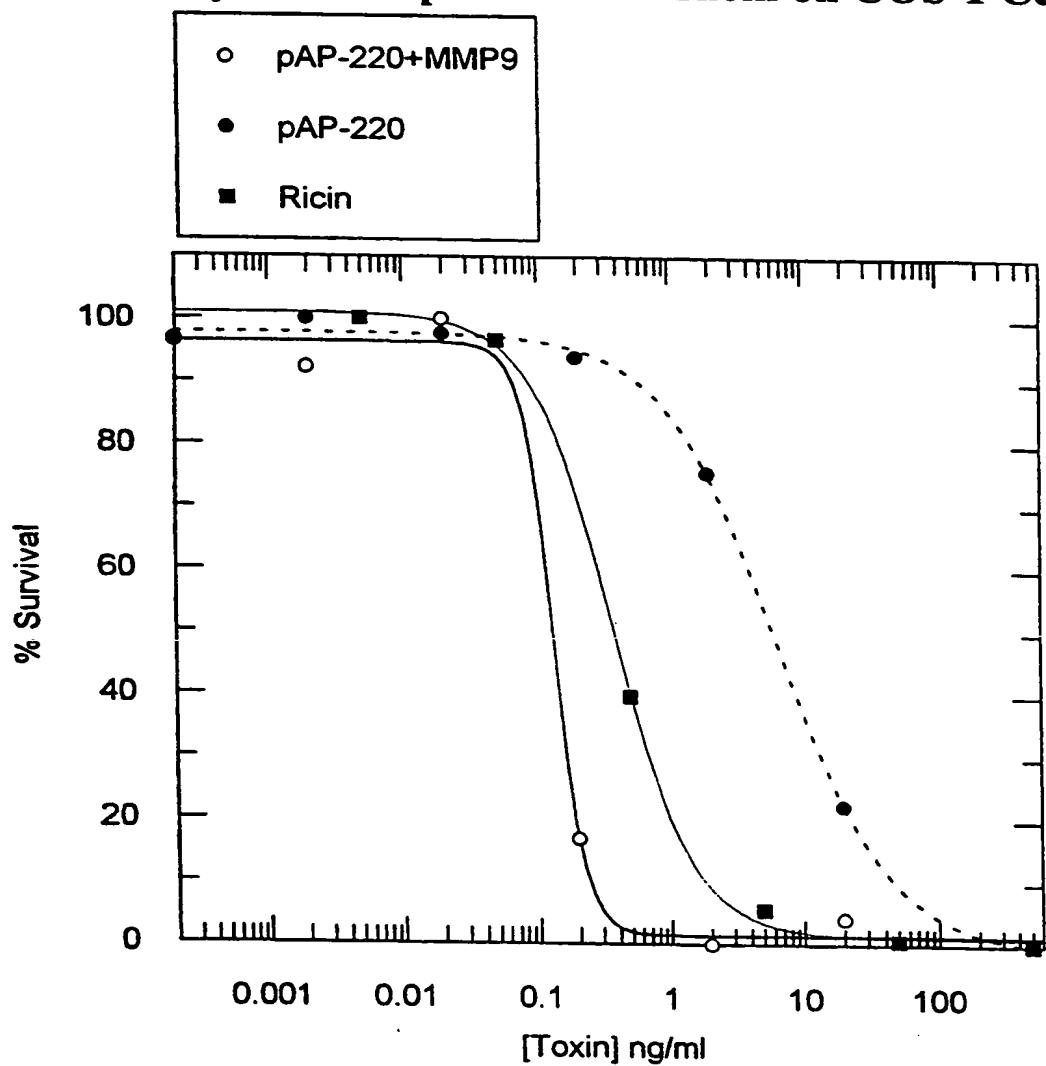
- A. 100 ng of pAP-256 variant
- B. 14.2 ng of pAP-256 variant
- C. 2.0 ng of pAP-256 variant
- D. 291 pg of pAP-256 variant
- E. 41.7 pg of pAP-256 variant
- F. Negative control
- G. Ricin A chain
- H. 41.7 pg of pAP-256 digested with HAV 3C protease
- I. 291 pg of pAP-256 digested with HAV 3C protease
- J. 2.0 ng of pAP-256 digested with HAV 3C protease
- K. 14.2 ng of pAP-256 digested with HAV 3C protease
- L. 100 ng of pAP-256 digested with HAV 3C protease
- M. RNA ladder

243/254

FIGURE 56**Cytotoxicity of Digested and Undigested pAP 214 with Cathepsin B to COS-1 Cells**

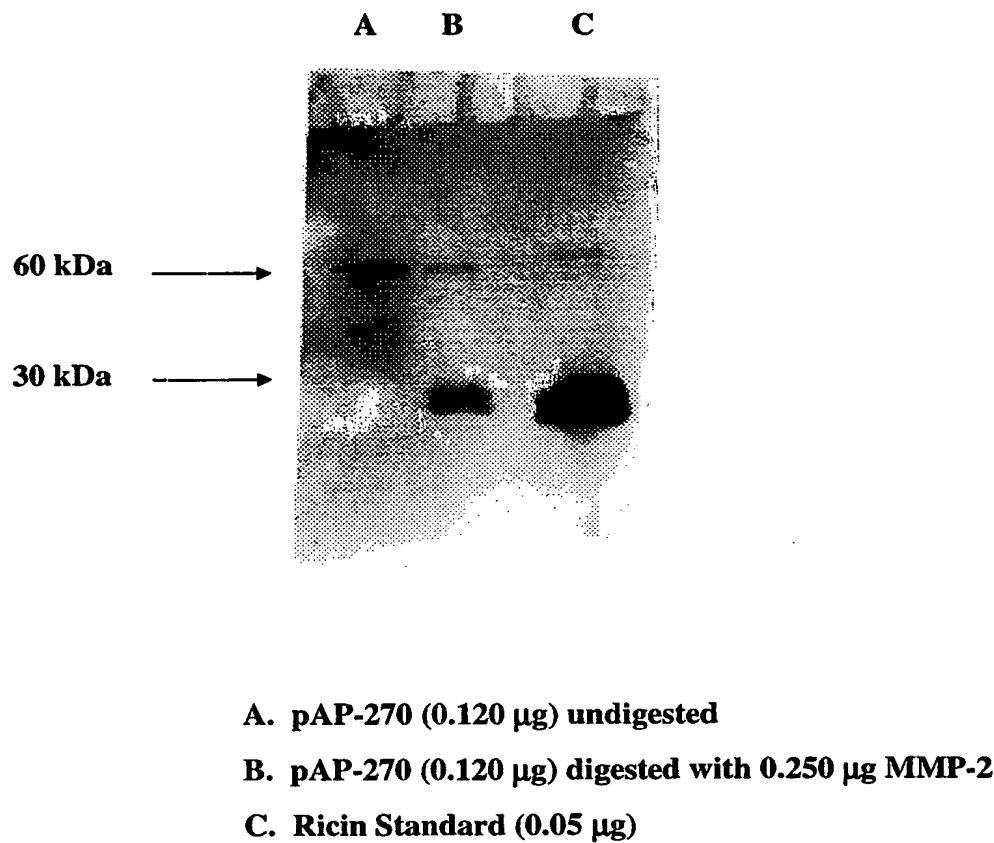
	Ricin	pAP 214	pAP 214 + Cathepsin B
IC ₅₀ (ng/ml)	0.11	1.9	0.078
Relative Toxicity	1X	17X	0.7X

244/254

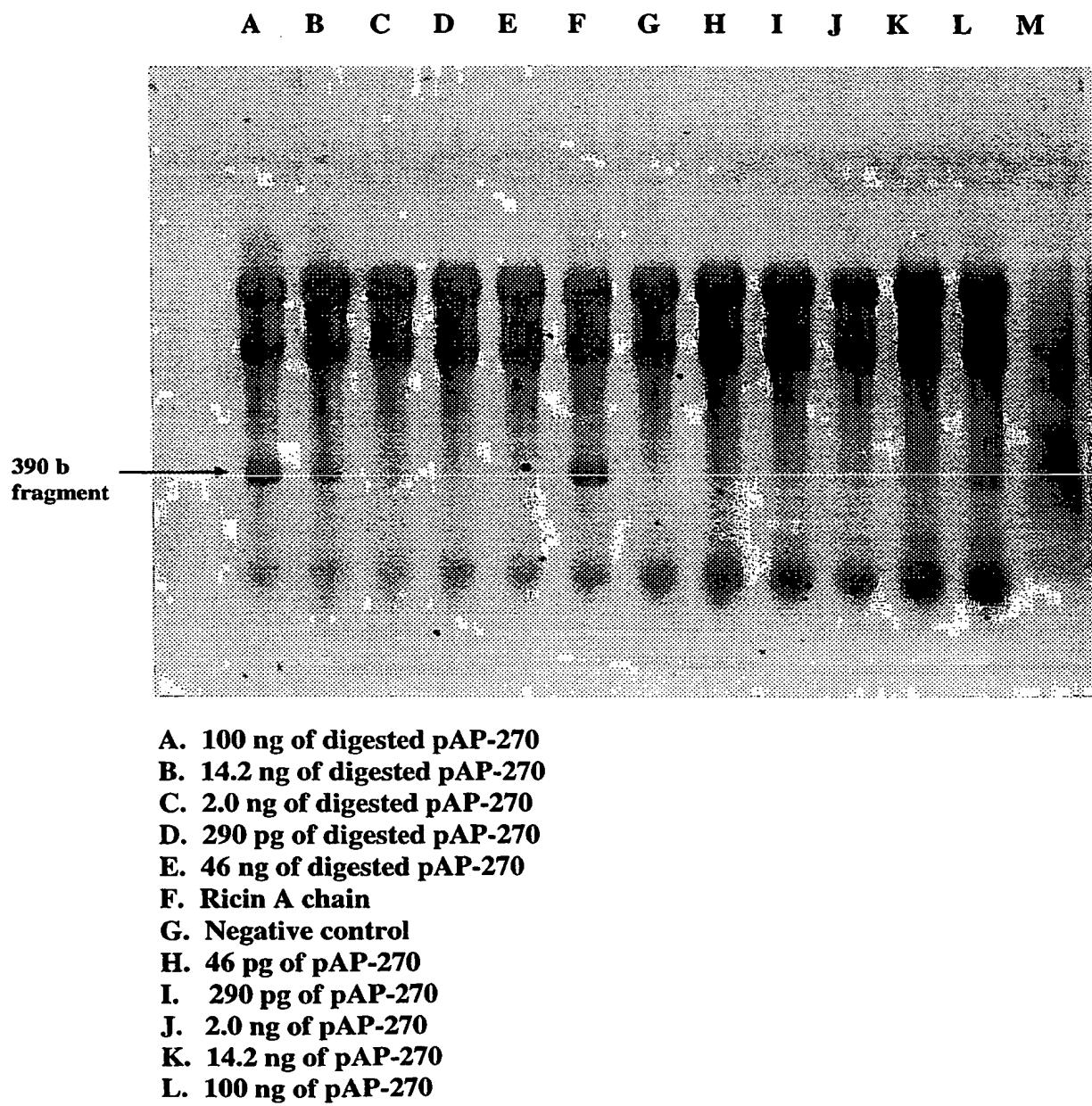
FIGURE 57**Cytotoxicity of pAP220 Digested with MMP-9 Compared to Freshly Thawed pAP220 and Ricin on COS-1 Cells**

	Ricin	pAP 220	pAP 220 + MMP-9
IC_{50} (ng/ml)	0.31	6.7	0.13
Relative Toxicity	1X	22X	0.4X

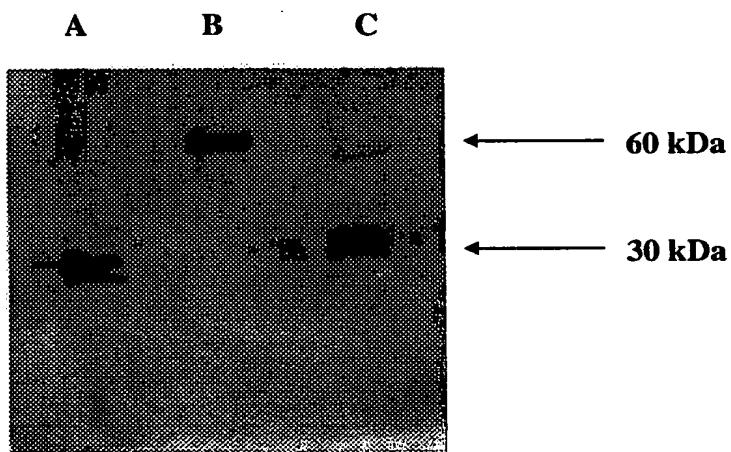
245/254

FIGURE 58**Cleavage of pAP-270 protein by The Matrix Metalloproteinase 2 (MMP-2)****SUBSTITUTE SHEET (RULE 26)**

246 / 254

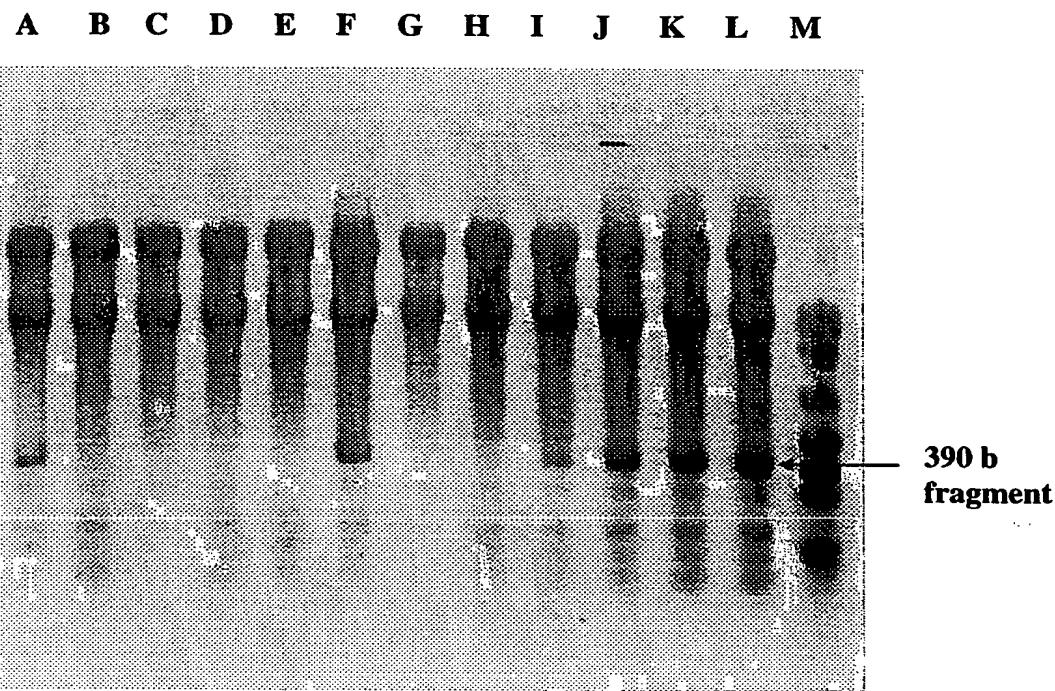
FIGURE 59**Activation of pAP-270 protein****SUBSTITUTE SHEET (RULE 26)**

247/254

FIGURE 60**Cleavage of pAP-288 protein by Plasminogen Tissue Activator (t-PA)**

- A. Ricin Standard (0.05µg)
- B. pAP-288 (0.66 µg) undigested
- C. pAP-288 (0.60 µg) digested with 0.18 µg of t-PA protease

248/254

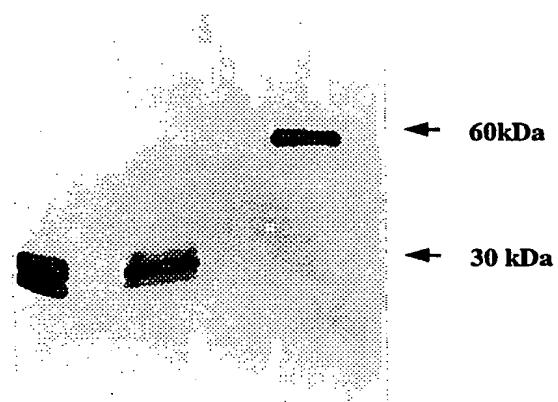
FIGURE 61**Activation of pAP-288 protein**

- A. 200 ng of pAP-288
- B. 28.4 ng of pAP-288
- C. 4.0 ng of pAP-288
- D. 482 pg of pAP-288
- E. 83.4 pg of pAP-288
- F. Ricin A chain
- G. Negative control
- H. 83.4 pg of pAP-288 digested with tissue Plasminogen Activator (t-PA)
- I. 482 pg of pAP-288 digested with t-PA
- J. 4.0 ng of pAP-288 digested with t-PA
- K. 28.4 ng of pAP-288 digested with t-PA
- L. 200 ng of pAP-288 digested with t-PA
- M. RNA ladder

249/254

FIGURE 62

Cleavage of pAP 294 With Human Neutrophil Elastase

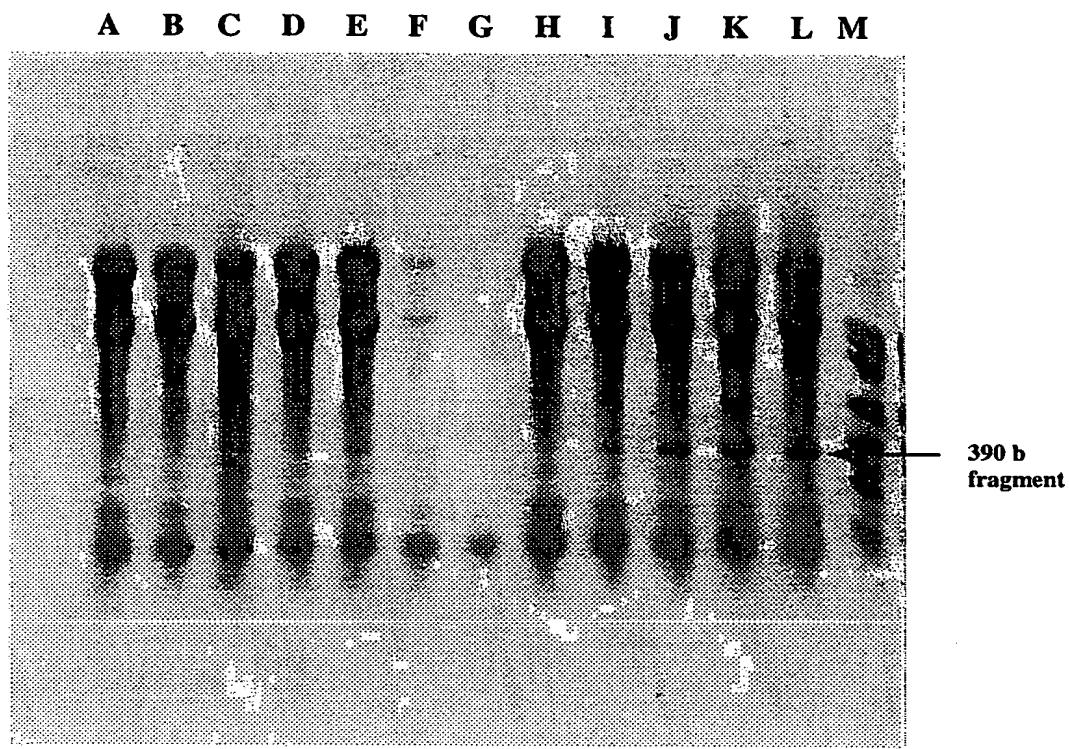


- A. Ricin Standard (0.050 µg)
- B. pAP 294 protein (0.171 µg) digested with 1.42 µg of Human Neutrophil Elastase
- C. pAP 294 protein (0.121 µg)

250/254

FIGURE 63

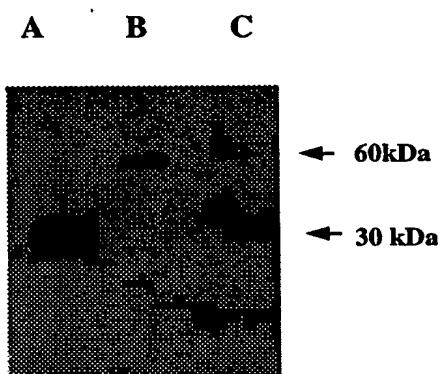
Activation of pAP 294 Protein



- A. 60 ng of pAP 294
- B. 8.57 ng of pAP 294
- C. 1.22 ng of pAP 294
- D. 175 pg of pAP 294
- E. 25 pg of pAP 294
- F. Ricin A chain
- G. Negative Control
- H. 360 ng of pAP 294 digested with Human Neutrophil Elastase
- I. 51 ng of pAP 294 digested with Human Neutrophil Elastase
- J. 7.3 ng of pAP 294 digested with Human Neutrophil Elastase
- K. 1.0 ng of pAP 294 digested with Human Neutrophil Elastase
- L. 150 pg of pAP 294 digested with Human Neutrophil Elastase
- M. RNA ladder

SUBSTITUTE SHEET (RULE 26)

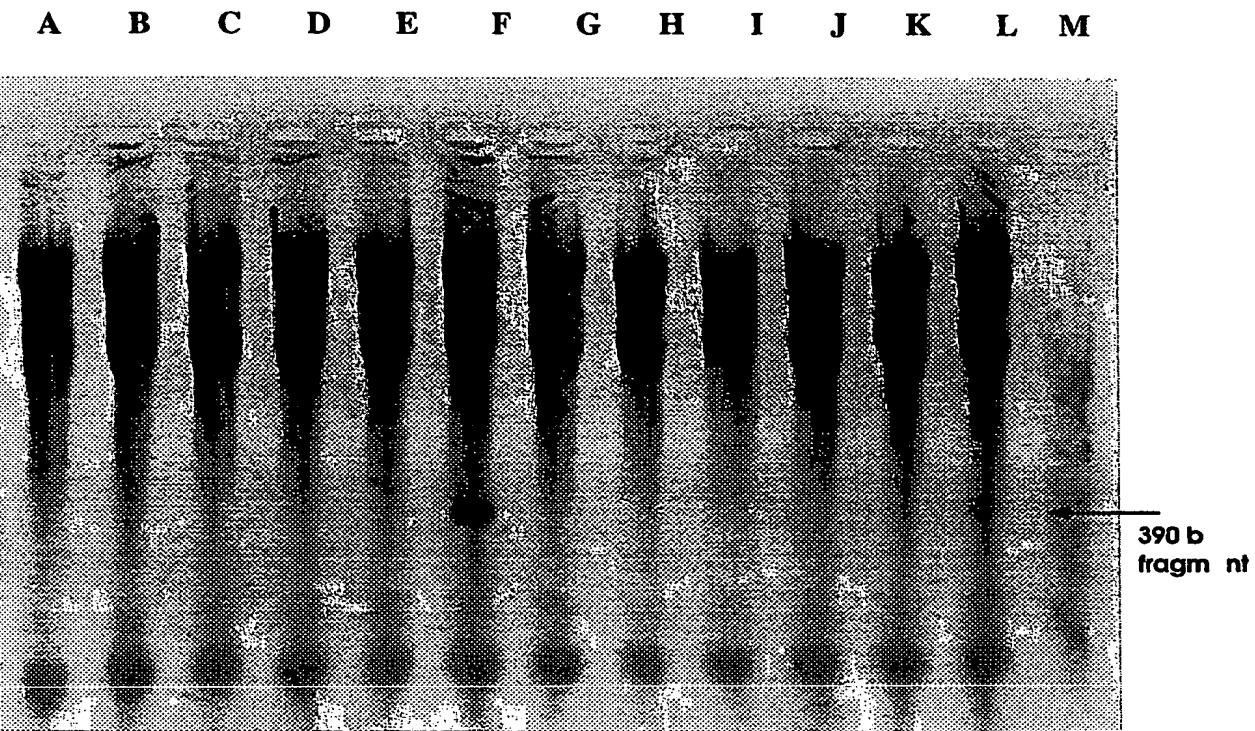
251/254

FIGURE 64**Cleavage of pAP 296 with Calpain**

- A. Ricin Standard (0.05 µg)
- B. pAP 296 (0.761 µg) undigested
- C. pAP 296 (0.761 µg) digested with 4.0 µg of Calpain

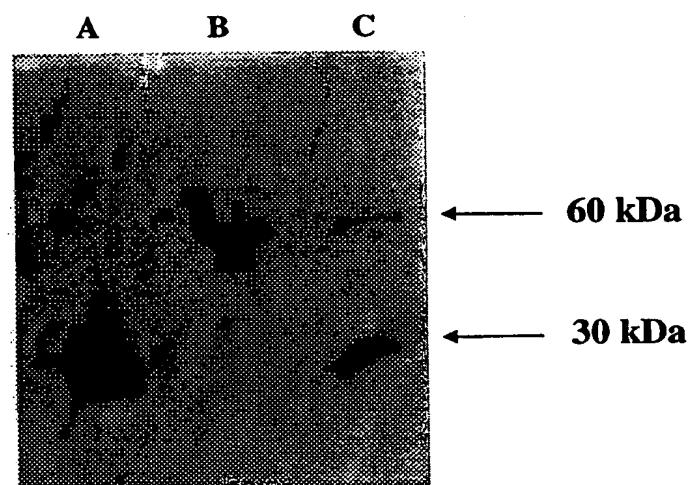
SUBSTITUTE SHEET (RULE 26)

252/254

FIGURE 65**Activation of pAP 296 Protein**

- A. 100 ng of pAP 296 variant
- B. 14.2 ng of pAP 296 variant
- C. 2.0 ng of pAP 296 variant
- D. 290 pg of pAP 296 variant
- E. 46 pg of pAP 296 variant
- F. Ricin A chain
- G. Negative control
- H. 46 pg of pAP 296 variant digested with Calpain
- I. 290 pg of pAP 296 variant digested with Calpain
- J. 2.0 ng of pAP 296 variant digested with Calpain
- K. 14.2 ng of pAP 296 variant digested with Calpain
- L. 100 ng of pAP 296 variant digested with Calpain
- M. RNA ladder

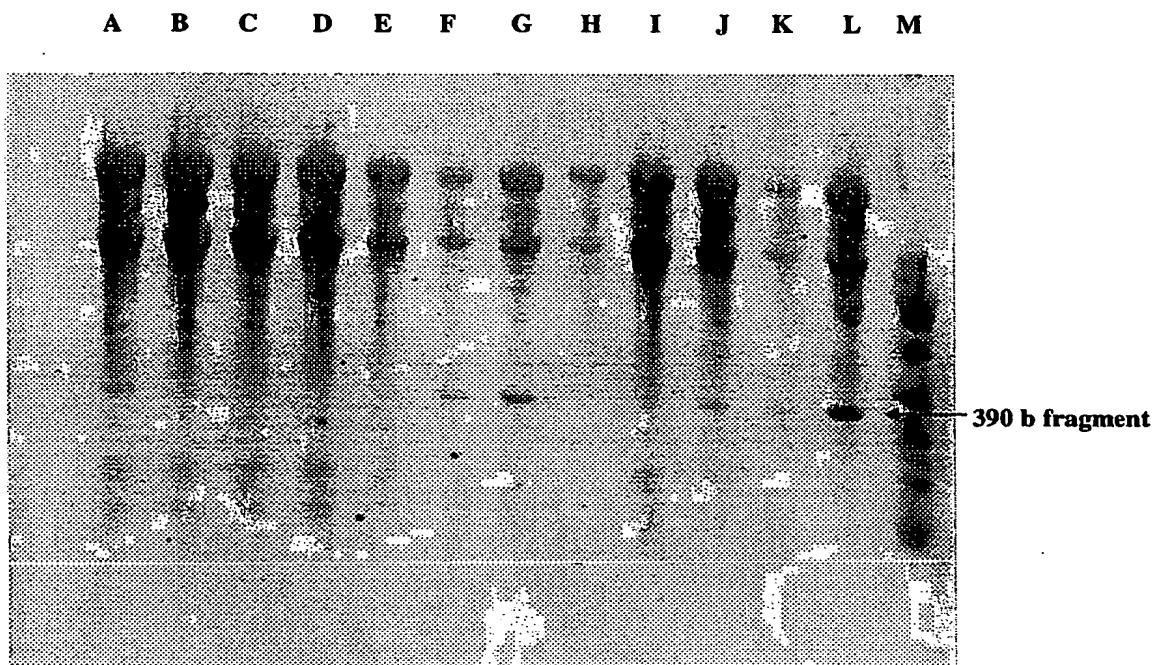
253/254

FIGURE 66**Cleavage of pAP-222 Protein by The Matrix Metalloproteinase 2 (MMP-2)**

- A. Ricin Standard (0.250 ug)
- B. pAP-222 Protein (0.250 ug)
- C. pAP-222 protein (0.250 ug) digested with 0.28 ug of MMP-2

SUBSTITUTE SHEET (RULE 26)

254/254

FIGURE 67**Activation of pAP-222 Protein**

- A. 100 ng of pAP-222 variant
- B. 14.2 ng of pAP-222 variant
- C. 2.0 ng of pAP-222 variant
- D. 291 pg of pAP-222 variant
- E. 41.7 pg of pAP-222 variant
- F. Ricin A chain
- G. Ricin A chain
- H. 41.7 pg of pAP-222 digested with MMP-2
- I. 291 pg of pAP-222 digested with MMP-2
- J. 2.0 ng of pAP-222 digested with MMP-2
- K. 14.2 ng of pAP-222 digested with MMP-2
- L. 100 ng of pAP-222 digested with MMP-2
- M. RNA ladder



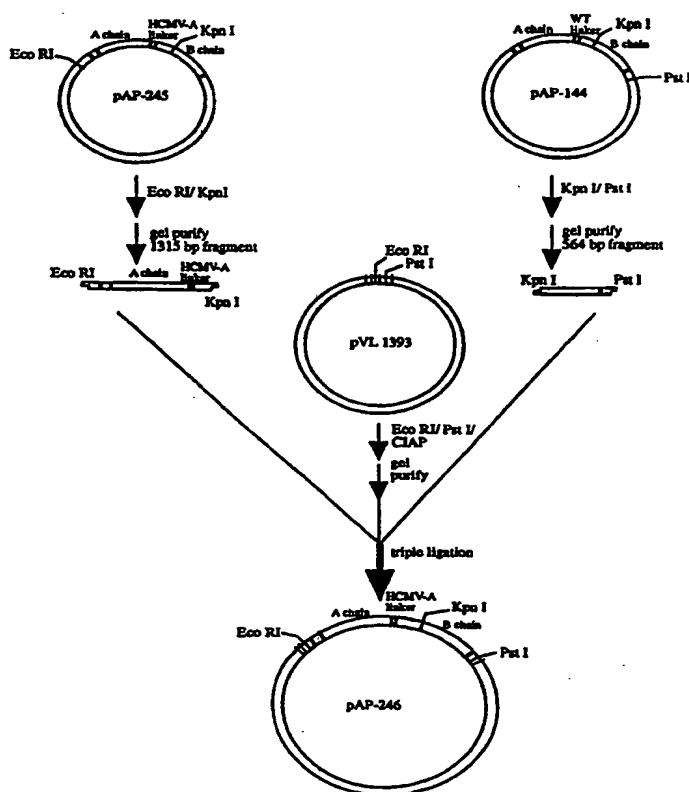
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/29, 15/62, 15/70, 15/86, A61K 38/16, 48/00		A3	(11) International Publication Number: WO 98/49311
			(43) International Publication Date: 5 November 1998 (05.11.98)
(21) International Application Number: PCT/CA98/00394		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 30 April 1998 (30.04.98)			
(30) Priority Data: 60/045,148 30 April 1997 (30.04.97) US 60/063,715 29 October 1997 (29.10.97) US			
(71) Applicant (for all designated States except US): DE NOVO ENZYME CORPORATION [CA/CA]; #2 Suite SFU Discovery Park, Burnaby, British Columbia V5A 1S6 (CA).			
(72) Inventor; and			
(75) Inventor/Applicant (for US only): BORGFORD, Thor [CA/CA]; 443 Fadar Street, New Westminster, British Columbia V3L 3T2 (CA).			
(74) Agent: BERESKIN & PARR; 40th floor, 40 King Street West, Toronto, Ontario M5H 3Y2 (CA).			

(54) Title: RICIN-LIKE TOXIN VARIANTS FOR TREATMENT OF CANCER, VIRAL OR PARASITIC INFECTIONS

(57) Abstract

The present invention provides a protein having an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains. The linker sequence contains a cleavage recognition site for a disease specific protease such as a cancer, fungal, viral or parasitic protease. The invention also relates to a nucleic acid molecule encoding the protein and to expression vectors incorporating the nucleic acid molecule. Also provided is a method of inhibiting or destroying mammalian cancer cells, cells infected with a virus, a fungus, or parasite, or parasites utilizing the nucleic acid molecules and proteins of the invention and pharmaceutical compositions for treating human cancer, viral infection, fungal infection, or parasitic infection.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Larvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

INTERNATIONAL SEARCH REPORT

Int'l Application No
PCT/EP 98/00394

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/29 C12N15/62 C12N15/70 C12N15/86 A61K38/16
A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 94 18332 A (US HEALTH) 18 August 1994 * see the whole document, esp. p.16 1.30 – p.20 1.2 * --- WESTBY M ET AL: "PREPARATION AND CHARACTERIZATION OF RECOMBINANT PRORICIN CONTAININGAN ALTERNATIVE PROTEASE-SENSITIVE LINKER SEQUENCE" BIOCONJUGATE CHEMISTRY, vol. 3, no. 5, 1 January 1992, pages 375-381, XP000578216 cited in the application * see the whole document, esp. last paragraph * --- -/-	1-10, 12-23, 25-35
Y		1-10, 12-23, 25-35

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

27 October 1998

10/11/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.
Fax: (+31-70) 340-3016

Authorized officer

Kania, T

INTERNATIONAL SEARCH REPORT

atational Application No
PCT/CA 98/00394

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LEPPLA S H ET AL: "DEVELOPMENT OF ANTHRAX-TOXIN BASED FUSION PROTEINS FOR TARGETING OF HIV-1-INFECTED CELLS" ZENTRALBLATT FUER BAKTERIOLOGIE. SUPPLEMENT, vol. 24, 1994, pages 431-442, XP002041056 cited in the application * see the whole document, esp. pp.437-39 * ----	1-35
A	COOK J P ET AL: "BIOLOGICALLY ACTIVE INTERLEUKIN 2-RICIN A CHAIN FUSION PROTEINS MAY REQUIRE INTRACELLULAR PROTEOLYTIC CLEAVAGE TO EXHIBIT A CYTOTOXIC EFFECT" BIOCONJUGATE CHEMISTRY, vol. 4, no. 6, 1 November 1993, pages 440-447, XP000417282 see the whole document	1-35
A	O'HARE M ET AL: "CYTOTOXICITY OF A RECOMBINANT RICIN-A-CHAIN FUSION PROTEIN CONTAINING A PROTEOLYTICALLY-CLEAVABLE SPACER SEQUENCE" FEBS LETTERS, vol. 273, no. 1/02, 29 October 1990, pages 200-204, XP002041057 cited in the application see the whole document	1-35
A	PANCHAL R. ET AL.: "Tumor protease-activated, pore-forming toxins from a combinatorial library" NATURE BIOTECHNOLOGY, vol. 14, no. 7, 14 July 1996, pages 852-856, XP002082096 cited in the application see the whole document	1-35
A	EP 0 466 222 A (DOWELANCO) 15 January 1992 cited in the application see the whole document	1-35
P,X	WO 97 41233 A (NOVO ENZYME CORP DE ;BORGFORD THOR (CA)) 6 November 1997 see the whole document	1-10, 12-23, 25-35

INTERNATIONAL SEARCH REPORT

.erbal application No.

PCT/CA 98/ 00394

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 25-30, 32, 33 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.

PCT/CA 98/00394

Patent document cited in search report		Publication date		Patent family member(s)		Publication date
WO 9418332	A	18-08-1994		US 5591631 A US 5677274 A AT 169959 T AU 682500 B AU 6392294 A DE 69412593 D EP 0684997 A		07-01-1997 14-10-1997 15-09-1998 09-10-1997 29-08-1994 24-09-1998 06-12-1995
EP 0466222	A	15-01-1992		US 5248606 A AU 638133 B AU 7832991 A CA 2044201 A CN 1062172 A JP 4279599 A US 5635384 A US 5646026 A		28-09-1993 17-06-1993 12-12-1991 12-12-1991 24-06-1992 05-10-1992 03-06-1997 08-07-1997
WO 9741233	A	06-11-1997		AU 2377097 A		19-11-1997